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Research Article

Tissue Distribution and Nutritional Regulation of Four Cholesterol Transport-Related Genes in Tiger Puffer (*Takifugu rubripes*)

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This study characterized four cholesterol transport-related genes, namely, *lcat*, *acat1*, *acat2*, and *mttp*, in juvenile tiger puffer in terms of tissue distribution (eye, heart, brain, skin, liver, spleen, muscle, and intestine) and nutritional regulation. Three feeding trials were conducted: (i) using diets with different cholesterol levels (0.11%, 0.65%, 1.10%, 2.32%, and 4.59% of dry matter); (ii) using diets with different lipid levels (8.05%, 12.02%, and 16.36% of dry matter); and (iii) a 1-month starvation experiment with different sampling times (Days 1, 4, 9, 16, and 31). The *lcat*, *acat1*, and *acat2* were the most abundantly expressed in the liver, while *mttp* was the most abundantly expressed in the intestine. The *lcat* had a medium expression level in the muscle and skin but the lowest expression level in the spleen and intestine. The *acat1* and *acat2* had similar tissue distribution patterns, except that *acat2* had a lower expression level in the heart but a higher level in the intestine than *acat1*. The *mttp* had medium expression levels in the liver and spleen but had the lowest level in the eye and skin. Different dietary cholesterol levels did not significantly affect the expression of these cholesterol transport genes in the liver and intestine, except that higher cholesterol levels (1.10%–4.59%) significantly downregulated the *acat2* expression in the liver. Dietary lipid levels also had a mild influence on the hepatic expression of these genes, except that the diet with 12.02% lipid resulted in significantly higher *acat1* expression than the one with 16.36% lipid. The starvation time significantly affected the hepatic expression of these genes. Long-term starvation (16 and 31 days) generally upregulated the hepatic gene expression of *lcat*, *acat1*, and *mttp* but generally downregulated that of *acat2*. This study provided preliminary knowledge about the tissue distribution and nutritional regulation of cholesterol transport-related genes in marine teleost.

1. Introduction

The vertebrates are able to biosynthesize cholesterol, and therefore, cholesterol is generally assumed to be a nonessential ingredient in their diets [1–3]. However, due to the fact that the proportion of cholesterol-rich fishmeal and fish oil is decreasing in fish diets, whether it is necessary to supplement exogenous cholesterol in fish feeds is becoming controversial [4, 5]. Some studies have suggested that the supplementation of cholesterol in low-fish or nonfish diets was beneficial to fish growth [1, 6–10]. It was assumed that the exogenous cholesterol supplementation spared the energy to be used for endogenous cholesterol biosynthesis. The previous studies on cholesterol in farmed fish mostly focused on the necessity of exogenous cholesterol supplementation in the diet [11–13]. However, the metabolism of cholesterol in farmed fish has seldom been investigated, although it is assumed that most processes of cholesterol metabolism could be conserved between fish

and mammals. In particular, regarding cholesterol metabolism, compared to cholesterol biosynthesis and conversion [1, 14, 15], even less studies have been conducted on cholesterol transport.

With tiger puffer, an important marine aquaculture fish species, this study aimed at preliminarily investigating the expression and functional characteristics of four cholesterol transport-related genes in terms of tissue distribution and nutritional adaptation. The lecithin cholesterol acyltransferase (Lcat) transfers fatty acids in the sn-2 position of phosphatidylcholine to unesterified cholesterol, thus forming lysophosphatidylcholine and cholesterol esters. The Lcat is synthesized in the liver and functions in the blood, where it preferentially binds to the high-density lipoproteins (HDL) and plays key roles in HDL maturation [16, 17]. The cholesterol acyltransferase (Acat) uses long-chain fatty acylcoenzyme A and cholesterol to biosynthesize cholesterol ester. Two *acat* genes, namely, *acat1* and *acat2*, have been identified in humans and mammals, which may function in different and complementary ways [18, 19]. The microsomal triglyceride transfer protein (Mttp) is critically involved in the formation and secretion of apolipoprotein B-containing lipoproteins, in particular the very low-density lipoprotein (VLDL) in the liver and the chylomicrons in the intestine [20].

The distribution of these cholesterol transport-related genes in different tissues has been investigated in human, swine, mouse, and zebrafish [21–26]. In contrast, little information has been available about the nutritional regulation of these genes. Previous studies with mammals have found that dietary cholesterol levels, fat levels, and fatty acid types all affect the cholesterol transport processes in species-specific ways [27–30]. In fish, both topics have been less studied. Limited studies in this area have shown that dietary the cholesterol level, lipid level, and source of lipid and protein in diets regulated the cholesterol transport process, but relevant studies on the proteins involved in the cholesterol transport process mainly focus on apolipoproteins (Apo), and a small number of studies investigated the regulation of *mttp* [31–34]. Very few fish studies have been available regarding the dietary effects on *lcat, acat1*, and *acat2*. The present study was aimed at preliminarily characterizing four cholesterol transport-related genes, *lcat, acat1, acat2*, and *mttp*, in terms of tissue distribution and nutritional regulation. For the nutritional regulation study, samples from our previous feeding trials regarding different nutritional factors, which are closely related to cholesterol metabolism, namely, dietary cholesterol level [35], dietary lipid level, and starvation time [36], were used in this study. The present results could provide basic knowledge about cholesterol transport in marine teleost.

2. Materials and Methods

2.1. Samples for the Tissue Distribution Study. Eight tissues of tiger puffer, namely, eye (E), heart (H), brain (B), skin (SK), liver (L), spleen (SP), muscle (M), and intestine (I) were collected as previously described [37]. Briefly, 15 juvenile fish (approximately weighing 20 g) were used, and the samples from five fish were pooled as a replicate. The collected samples were immediately put into liquid nitrogen and then transferred to −76°C storage before use. Fish were first anesthetized with eugenol before being sampled. All sampling handlings and all fish-rearing protocols in this study were reviewed and approved by the Animal Care and Use Committee of the Yellow Sea Fisheries Research Institute.

2.2. Samples for the Nutritional Regulation Study. For the nutritional regulation study, samples from our previous feeding experiments were used in this study. In the first experiment [35], juvenile tiger puffers with an initial body weight of 12 g were fed experimental diets with different cholesterol levels (0.11%, 0.65%, 1.10%, 2.32%, and 4.59% of dry matter) for 10 weeks. These diets contained 30% fishmeal without fish oil. The five diets were named control (CHO-0), CHO-0.5, CHO-1.0, CHO-2.0, and CHO-4.0, respectively. Each experimental diet was fed to three tanks (polyethylene, 200 L, 30 tiger puffer/tank). Fish were hand-fed three times (6:30/12:30/18:30) each day to apparent satiety. Fish were cultured in flow-through seawater. The liver and intestine samples from six fish/tank were collected at the end of the feeding experiment.

In the second experiment, juvenile tiger puffers with an average initial weight of 19.5 g were fed experimental diets with different lipid levels (8.05%, 12.02%, and 16.36% of dry matter) for 9 weeks, followed by a fasting duration of 1 month [36]. The experimental diets were named LL (control, low lipid), ML (medium lipid), and HL (high lipid), respectively. The feeding experiments were performed in cages put in cement tanks (1.4 m × m 1.4 m × 1.0 m; 50 tiger puffer/cage). Flow-through seawater was used. Each experimental diet was fed to six cages. During the feeding trial, fish were fed by hand to apparent satiety two times daily (6:00/18:00). At the end, liver samples from six fish/cage were randomly collected. After the feeding was ended, 30 randomly selected juveniles/cage of the control (LL) group (8.05% dietary lipid) were starved for 1 month (31 days). During the 1-month starvation, samples of liver from three fish/cages were collected at five time-points, i.e., 1, 4, 9, 16, and 31 days after the start of starvation. The ending day of the feeding trial was named day 0 (S0), and the other time points were designated as S1, S4, S9, S16, and S31, respectively.

2.3. qRT-PCR. The total RNA in all tissue samples was firstly extracted using the kit TaKaRa RNAiso Plus and then reversely transcribed with Evo M-MLVRT Mix Kit with gDNA Clean for qPCR (Accurate Biotechnology Co., Ltd., China) following the user's instructions. For the tissue distribution study, three pooled samples of each tissue based on 15 fish (five in each pool) were used for the RNA extraction. For the feeding studies with different cholesterol levels or different lipid levels, pooled tissue samples of six fish per tank were used, and for the starvation study, pooled tissue samples of three fish per cage at each time point were used for the RNA extraction.

The qRT-PCR was conducted to analyze the mRNA expression of *lcat, acat1, acat2*, and *mttp* across different fish tissues, as well as their gene expression in response to different nutritional status. Specific primers (Table 1) for the four genes were designed using NCBI and synthesized by a commercial company (TsingKe, Qingdao, China). According to our previous screening

Table 1: Primer information.

Primer	Sequence (5′–3′)	GenBank reference	PL (bp)
lcat-F	TGACTATGAGGACGGGTGGT	XM_003977753.3	78
lcat-R	GTGTTGTCCCCATCAGCGTA		
mttp-F	TCTCGCATAACTACGACCGC	XM_011612378.2	73
mttp-R	GCCGATTGTGCCATGAATCC		
acat1-F	TTGGGTTCGGTTGTGAAT	XM_003976083.3	114
acat1-R	GAGGCAGATGGAGGTGGT		
acat2-F	ACGCCTCAGGTATGAACGAC	XM_003971888.3	186
acat2-R	GTTTTACGCCACGCTTCTCG		
β-actin-F	CCAGAAAGACAGCTACGTTGG	U37499.1	147
β-actin-R	GCAACTCTCAGCTCGTTGTAG		
EF1-α-F	GTAGGTGATGAAGCCCAGAGCA	MT023044.1	204
EF1-α-R	CTGGGTCATCTTCTCCCTGT		

Abbreviation: PL, product length.

[36], β-actin and EF1α were suitable house-keeper genes for this study. The amplification efficiency (E value) of all primers was examined by drawing standard curves (6-step 4-fold dilution, E: 95%–105%). The linear regression coefficients (R^2) were greater than 0.99. The detailed qRT-PCR procedures were the same as our previous descriptions [36; 37]. SYBR Green Premix Pro Taq HS qPCR Kit II (Accurate Biotechnology (Hunan) Co. Ltd., Hunan, China) and a quantitative thermal cycler (Roche LightCycler 96, Basel, Switzerland) were used for the real-time qPCR. The reaction system consists of 2 μL cDNA template, 10 μL SYBR Green Pro Taq HS Premix II, 0.8 μL forward primer (10 μM), 0.8 L reverse primer (10 μM), and 6.4 μL sterilized water. The program was as follows: 95°C for 30 s followed by 40 cycles of "95°C for 5 s, 57°C for 30 s, 72°C for 30 s." Melting curve analysis (6.4°C increment/min from 65 to 97°C) was performed after the amplification phase for confirmation of the sole product. Each sample was run in triplicate. The relative mRNA expression levels were calculated according to the $2^{-\Delta\Delta CT}$ calculation method [38].

2.4. Statistics. One-way analysis of variance (one-way ANOVA, SPSS 25.0, SPSS Inc. Chicago, USA) was used in the statistics of this study. Tukey's multiple range test was used to determine the significant ($P < 0.05$) differences between means of different groups.

3. Results and Discussion

3.1. Tissue Distribution. The expression of these four genes was ubiquitous across all the examined tissues, although the expression level varied depending on gene type and tissue type. The *lcat* had the highest expression level in the liver, which was much higher than that in other tissues (Figure 1(a)). This result was consistent with the fact that *lcat* is primarily synthesized in the liver [17]. Medium expression levels of *lcat* were observed in the muscle and skin, but the lowest expression levels were observed in the intestine. This low expression level of *lcat* in the intestine of tiger puffer was different from what observed in pigs [26], which showed that the small intestine had a high *lcat* level. However, a study on mice also reported the low expression of *lcat* in the intestine [21]. The appropriate explanation for the differences in *lcat* expression levels among different animal species, according to the relevant available studies so far, could be that the function and expression of cholesterol transport genes may be correlated with the structure of the digestive tract of animals. The small intestine of a pig is about 15 times longer than its body length and is the most important digestive organ. However, the intestine of a tiger puffer, which is much shorter, has a totally different morphology and structure. To further definitively explain these differences, further research is needed.

The *acat* also had the highest expression level in the liver, followed by the brain, eye, and muscle but had the lowest highest expression level in the spleen (Figures 1(b) and 1(c)). In general, the two *acat* subtypes, *acat1* and *acat2*, had similar distribution patterns among tissues. However, differences were still observed. Compared to *acat1, acat2* had a lower expression level in the heart but a higher level in the intestine. Different functions of *acat1* and *acat2* in different tissues have been observed in humans. The two *acat* genes in mammals may function in different and complementary ways [18, 19]. It has been observed that in macrophages, skin cells, ovary cells, and adrenal cells, *acat1* is the primary isotype and constitutes more than 90% of the total activity of *acat*, while in the mucosal cells of the intestine, *acat2* is the primary isotype [24, 39]. It seems that the higher expression of *acat2* in the intestine was conserved between mammals and fish.

The tissue distribution pattern of *mttp* was largely different from the three genes mentioned above. The difference could be mainly due to the fact that *mttp* was not a gene specifically related to cholesterol metabolism. As a protein widely involved in the formation and secretion of ApoB-rich lipoproteins, Mttp plays important roles in much broader lipid metabolic pathways, in particular those related to chylomicrons and VLDL. That was why the *mttp* gene was highly expressed in mouse intestine and liver [22, 23]. Similarly in tiger puffer, the *mttp* had the highest expression level in the intestine, much higher compared with other tissues (Figure 1(d)). This could be due to the fact that the ApoB-

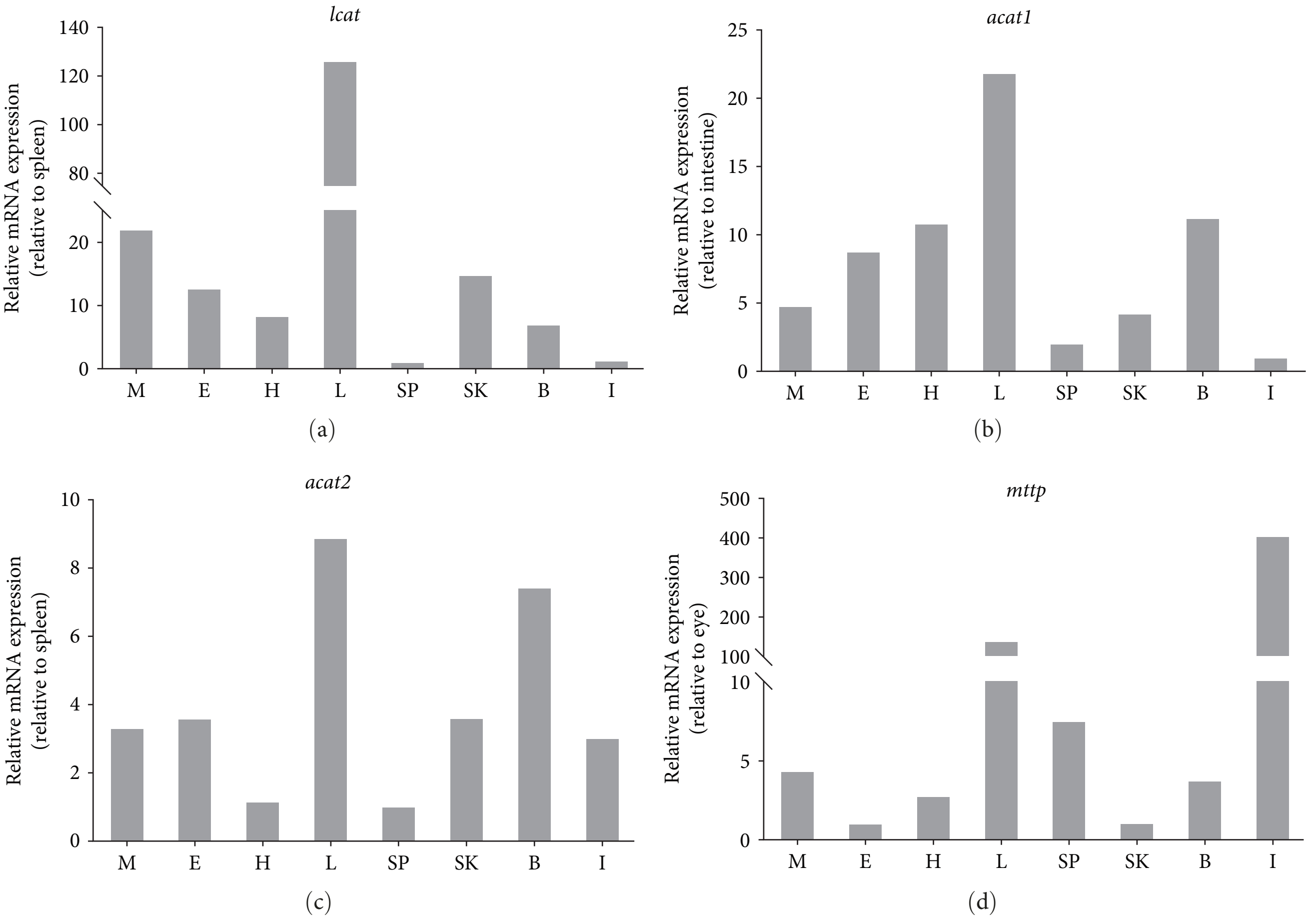

Figure 1: Tissue distribution of four cholesterol transport-related genes (*lcat* (a), *acat1* (b), *acat2* (c), and *mttp* (d)) in tiger puffer (*Takifugu rubripes*). Results were expressed as relative mRNA expression with respect to the lowest expression. The following tissues were analyzed: muscle (M), heart (H), eye (E), liver (L), spleen (SP), skin (SK), brain (B), and intestine (I). For the mRNA expression of these in various tissues, only means (without standard error) were presented due to the fact that large differences existed in intragroup variation among tissues.

rich lipoproteins are critically involved in the intestinal digestion and absorption of lipids [27, 40].

The liver of tiger puffer also had a high *mttp* expression level, about 100 times higher than that in the eye and skin, which had the lowest *mttp* expression level among the examined tissues. In another finfish species, blunt snout bream (*Megalobrama amblycephala*), the liver and intestine also had the highest *mttp* expression level, but the liver had a much higher expression level compared to the intestine [41]. In zebrafish, the anterior intestine had the highest *mttp* gene expression, followed by the posterior intestine and liver, which were much higher compared to other tissues [25]. Tiger puffer had a relatively unique lipid storage way. They store lipids mainly in the liver, making the liver more like a lipid storage site rather than a lipid metabolism center [42, 43]. This could partly explain why the tiger puffer liver had a lower *mttp* expression level compared to the intestine.

3.2. Regulation by Dietary Cholesterol Levels. In general, dietary cholesterol levels did not significantly affect the expression of all these cholesterol transport-related genes in the liver and intestine, except that higher cholesterol levels (1.10%–4.59%) significantly decreased the *acat2* expression in the liver ($P < 0.05$) (Figures 2 and 3). Actually, increasing dietary cholesterol levels also linearly downregulated the *acat1* expression in the liver, although no significant difference was observed due to the large intragroup variations. The downregulation of *acat1* and *acat2* expression in the liver could be associated with the function of Acat in dietary cholesterol absorption and cholesterol biosynthesis [44]. Acat1 catalyzes the formation of fatty acid-cholesterol esters, preferentially utilizing oleoyl-CoA ((9Z)-octadecenoyl-CoA) as substrate [44, 45]. Besides the roles in dietary cholesterol absorption, Acat2 is also involved in cholesterol biosynthesis [46]. In this study, excess dietary cholesterol supply exerted feedback regulation on the dietary cholesterol absorption and cholesterol biosynthesis, and subsequently resulted in downregulated expression of *acat1* and *acat2*. The biosynthesis of cholesterol in fish was sensitive to dietary cholesterol levels, and the inhibition of cholesterol synthesis in fish by excess dietary cholesterol has been widely observed [7, 8, 35, 47]. However, in rainbow trout (*Oncorhynchus mykiss*), dietary cholesterol supplementation of 0.9%–1.5% significantly upregulated the hepatic gene expression of *acat* compared to a

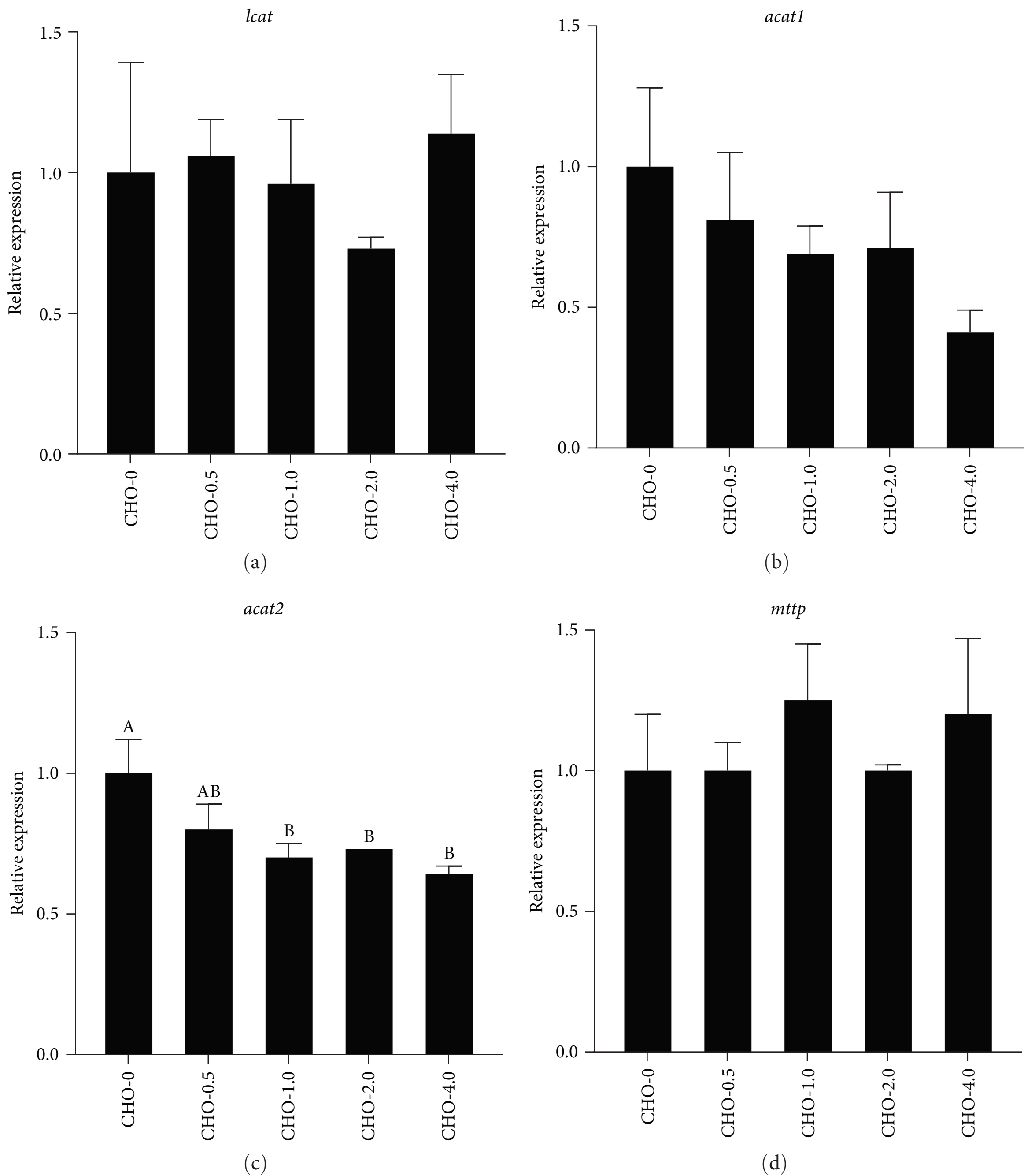

Figure 2: Effects of cholesterol levels in the diets on the gene expression related to cholesterol transport (*lcat* (a), *acat1* (b), *acat2* (c), and *mttp* (d)) in tiger puffer (*Takifugu rubripes*) liver (mean ± standard error). For a certain gene, data not sharing the same letter are significantly ($P < 0.05$) different.

lower supplementation level (0%–0.6%) [7]. This difference between different species could be mainly related to the cholesterol level in the diet. Stimulation of *acat* expression by a moderately high dietary cholesterol supplementation was also observed in chicks [48], guinea pigs [49], and rats [50]. In *in vitro* studies, it was also observed that the activity of ACAT can be increased by the addition of cholesterol to microsomal fractions [51, 52].

It was unexpected that the *lcat* and *mttp* expression was not influenced significantly ($P > 0.05$) by cholesterol levels in the diet. Lcat catalyzes the conversion of phosphatidylcholines and cholesterol to lysophosphatidylcholines and cholesteryl esters on the HDL and low-density lipoproteins (LDL) surface [17, 46, 53]. After that, the cholesterol esters are delivered back to the liver. Therefore, Lcat plays important roles in the reverse transport of cholesterol. The absence of significant influence of dietary cholesterol level on *lcat* and *mttp* expression could be related to the fact that tiger puffers have a high buffering capacity in maintaining a stable body cholesterol level [35].

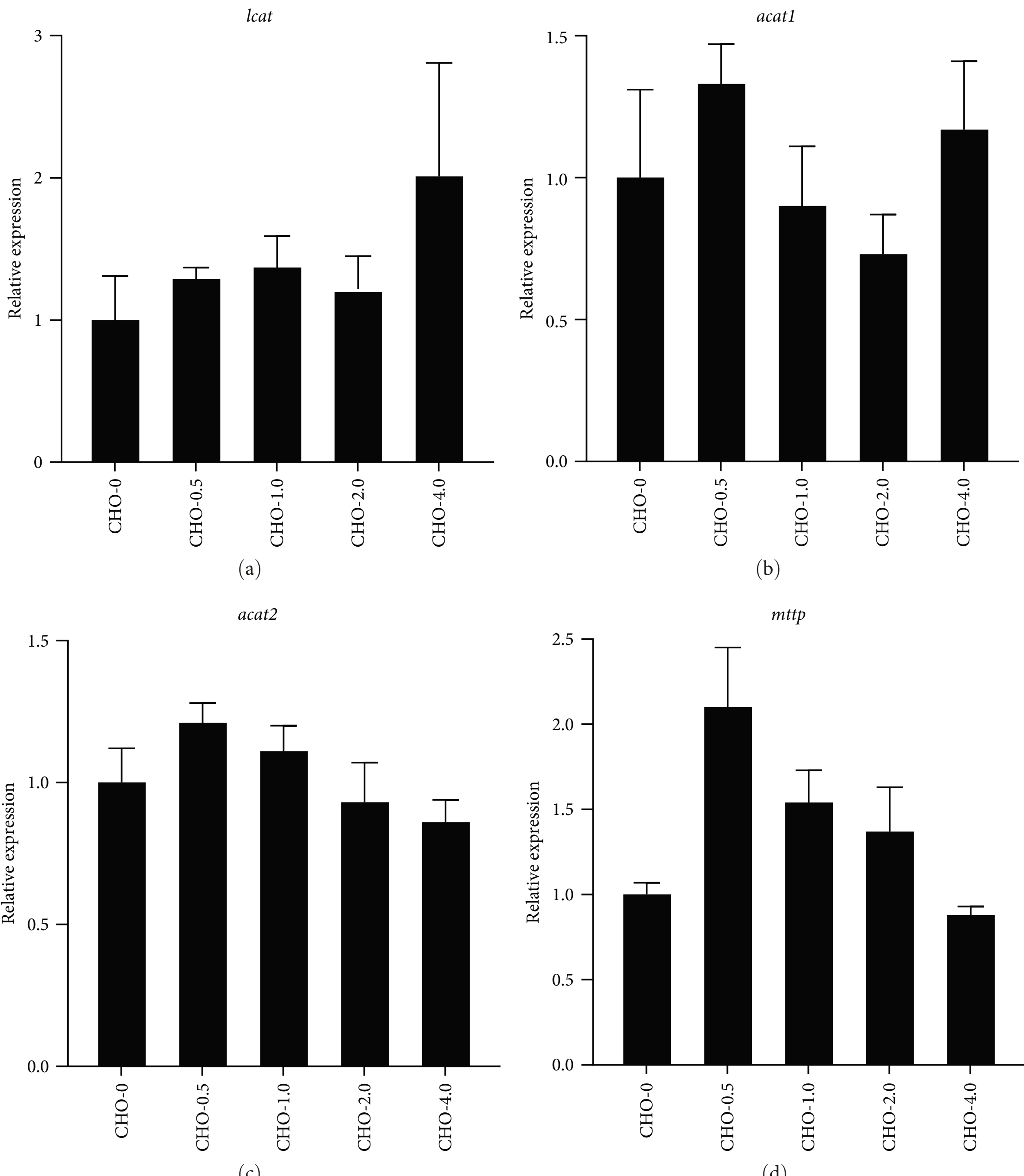

Figure 3: Effects of cholesterol levels in the diets on the gene expression related to cholesterol transport (*lcat* (a), *acat1* (b), *acat2* (c), and *mttp* (d)) in tiger puffer (*Takifugu rubripes*) intestine (mean ± standard error).

3.3. Regulation by Dietary Lipid Level. Dietary lipid levels also had a mild influence on the hepatic expression of these cholesterol transport-related genes, except that the diet with 12.02% lipid resulted in significantly ($P < 0.05$) higher *acat1* expression than the one with 16.36% lipid (Figure 4). Few studies have investigated the effects of dietary lipid levels on gene expression related to cholesterol transport. In adult male Sprague–Dawley rats, the LCAT mRNA level was relatively resistant to the changes in dietary fat content [21]. In channel catfish (*Ictalurus punctatus*), a high-lipid diet decreased the expression of *mttp* in the liver [34]. However, in contrast, in blunt snout bream, an elevation of dietary lipid content from 5% to 11% significantly upregulated the *mttp* mRNA abundance in the muscle, intestine, and liver [41]. A hepatic transcriptome assay on tiger puffer has shown that in tiger puffer, the lipid and cholesterol metabolism were not sensitive to the change of lipid level in the diet [54]. This may also be associated with the fact that the liver is more like a lipid storage organ of tiger puffer [42, 43]. Another explanation for this result was that the fatty acids contained in the dietary lipids

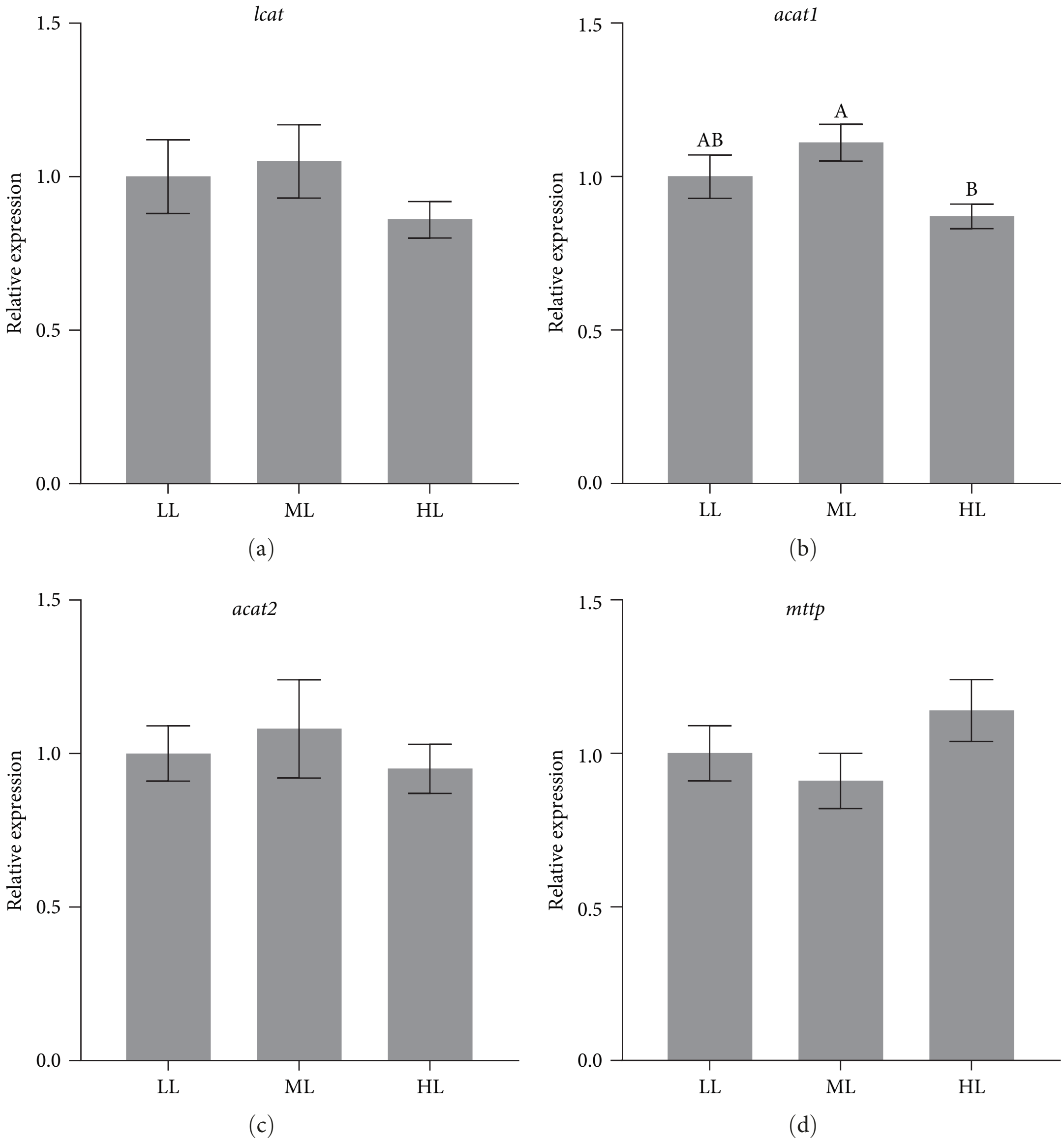

Figure 4: Effects of lipid levels in the diet on the gene expression related to cholesterol transport (*lcat* (a), *acat1* (b), *acat2* (c), and *mttp* (d)) in tiger puffer (*Takifugu rubripes*) liver (mean ± standard error). For a certain gene, data not sharing the same letter are significantly ($P < 0.05$) different.

had a low affinity to the proteins examined. Taking *lcat* as an example, a study on rats showed that the hepatic LCAT mRNA expression and serum LCAT activity were significantly higher in rats fed triolein compared with those fed tripalmitin or menhaden oil [28]. The lipid level grades in this study were reached by modulation of the level of fish oil, of which the fatty acids may have low affinity to Lcat. For *mttp*, a study on turbot (*Scophthalmus maximus*) also showed that fish-fed diets based on fish oil had significantly lower *mttp* expression than those fed the diet based on soybean oil or linseed oil [32, 33].

3.4. Regulation by Starvation. The starvation time significantly affected the hepatic expression of these cholesterol transport genes (Figure 5). Long-term starvation (16 and 31 days) generally upregulated the hepatic gene expression of *lcat, acat1*, and *mttp* but generally downregulated that of *acat2*. The stimulation of *lcat* expression by long-term starvation could be due to the compensatory requirement of HDL and LDL assembly during lipid mobilization for energy supply. Similarly, the stimulation of *mttp* by long-term starvation could be due to the compensatory increase of lipid absorption activity. In mice, starvation also induced the gene expression of *mttp* in liver cells [55]. However, in zebrafish larvae of 5–15 days post fertilization, feeding stimulated the gene expression of *mttp* compared to fasting [25].

It was interesting to observe that the response to starvation was different between *acat1* and *acat2*. Considering the concurrent upregulation of *cat, acat1*, and *mttp* expression by starvation, and the opposite response of the two *acat* subtypes to starvation, it is speculated that in tiger puffer *acat1* may be involved in dietary cholesterol absorption,

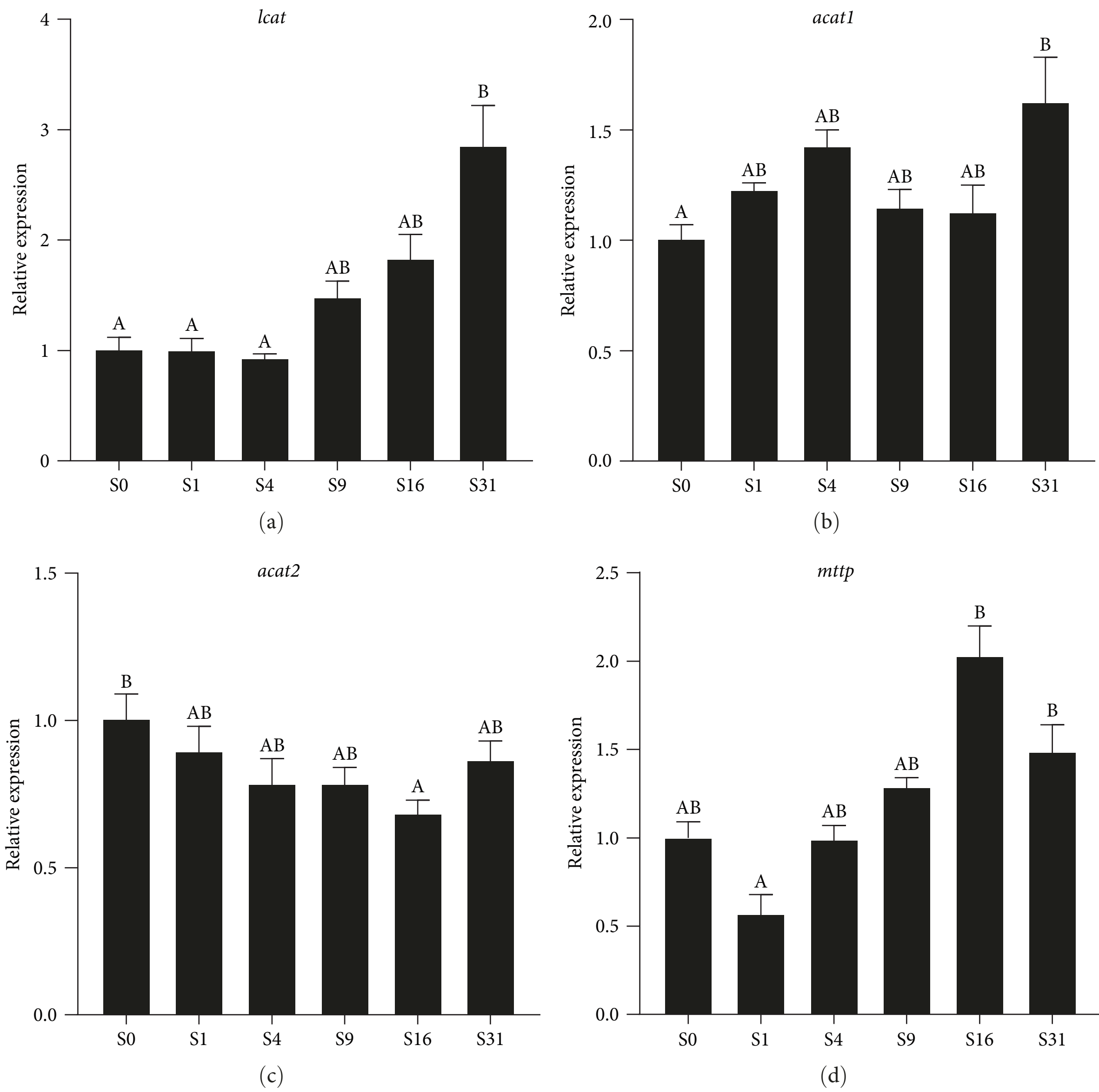

FIGURE 5: Effects of starvation period on the gene expression related to cholesterol transport (*lcat* (a), *acat1* (b), *acat2* (c), and *mttp* (d)) in tiger puffer (*Takifugu rubripes*) liver (mean ± standard error). For a certain gene, data not sharing the same letter are significantly ($P < 0.05$) different.

but *acat2* may be more closely involved in the cholesterol biosynthesis. A study on turbot has suggested the inhibition of cholesterol biosynthesis by starvation via inhibition of HMGR [47]. In humans and mammals, starvation led to lower plasma leptin concentrations, which further inhibited the cholesterol biosynthesis [56, 57]. Therefore, the inhibition of *acat2* expression may be a way by which starvation inhibits cholesterol biosynthesis in tiger puffer. More precise mechanisms involved in the cholesterol metabolism regulation by nutritional status warrant further studies.

4. Conclusions

The four cholesterol transport-related genes, *lcat, acat1, acat2*, and *mttp*, had higher expression levels in the liver and intestine, confirming their conserved functions in these tissues of tiger puffer. The *acat1* and *acat2* had similar tissue distribution patterns, expect that *acat2* had a lower expression level in the heart but a higher level in the intestine than *acat1*. Higher dietary cholesterol levels (1.10%–4.59%) significantly downregulated the *acat2* expression in the liver. The lipid levels in diet had mild influences on the hepatic expression of these genes. Long-term starvation (16 and 31 days) generally upregulated the hepatic gene expression of *lcat, acat1*, and *mttp* but generally downregulated that of *acat2*. The two *acat* subtypes, *acat1* and *acat2*, may have different functions in tiger puffer.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Acknowledgments

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