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MicroRNA expression profile and lipid metabolism characteristics in liver of rat undergoing high-fat diet

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Abstract: This study aimed to investigate the microRNA expression profile and the characteristics of lipid metabolism in the livers of rats undergoing a high-fat diet. Fifty male Sprague-Dawley (SD) rats were divided into a standard chow group (C group, N = 10) and a high-fat diet group (H group, N = 40). After 12 weeks, the rat body weight, body length, fat mass, and serum lipid concentration were measured. The expression profile of microRNAs and the gene and protein expression levels involved in lipid metabolism in rat liver were detected. Body fat and serum lipid concentrations were all significantly higher in the H group than those in the C group ($p < 0.05$ or $p < 0.01$). The expression of 10 microRNAs showed significant differences in the liver ($p < 0.05$). In particular, the let-7 family expression levels significantly increased ($p < 0.05$) in the H group compared with those in the C group. Compared with the C group, the high-fat diet resulted in low FAS, CPT1A, and ApoAI mRNA expression levels ($p < 0.05$ or $p < 0.01$) and high PPARα and FAT/CD36 mRNA expression levels in the H group rat liver ($p < 0.01$). Meanwhile, the protein PPARα, FAS, CPT1A, FAT/CD36, and ApoAI expression levels were all significantly lower in the H group than those in the C group ($p < 0.05$ or $p < 0.01$). In conclusion, the high-fat diet increased the body fat and serum lipid levels and altered the 10 microRNA expression levels in the liver. The high-fat diet may affect hepatic carbohydrate metabolism and increase ectopic fat accumulation through let-7 family overexpression. The high-fat diet for 12 weeks decreased lipid metabolism level in the liver, thereby decreasing fatty acid synthesis, oxidation, and transport by down-regulating the PPARα, FAS, CPT1A, FAT/CD36, and ApoAI protein levels.

Introduction

High-fat diet and lack of exercise are important factors leading to excessive body fat, liver fat accumulation, and lipid metabolic disorder. Lipid disorder, which has increased at an alarming rate worldwide, has become one of the most important medical concerns in the 21st century and increasingly threatens human health.

MicroRNAs are a kind of short (~22 nt) endogenous single-stranded regulatory RNAs, that were found in *Caenorhabditis elegans* in 1993 (Yates *et al*., 2013). MicroRNAs regulate the target gene expression level bycombining the 3' untranslated region with the target gene. MicroRNAs are involved in nearly all developmental and pathological processes in animals (Ha and Kim, 2014). The abnormal expression of microRNAs is related to a variety of diseases, such as various forms of cancer, congenital heart disease (Mukai *et al*., 2018), obesity (Kuryłowicz *et al*., 2018; Yu *et al*., 2018), and lipid metabolism (Aryal *et al*., 2017).

Given that the liver is a pivotal metabolic organ, hepatic impairment may have serious consequences. Metabolism imbalance of fatty acid, such as uptake, synthesis, oxidation, and secretion in the liver may lead to lipid accumulation and hepatic steatosis (Tilg and Moschen, 2010). The key factors and enzymes that regulate the metabolism of fatty acids play important roles in hepatic steatosis. Peroxisome proliferator-activated receptor α (PPARα) is one of the nuclear receptor superfamilies that belong to the transcription factors of ligand activation, which can be activated by fatty acids, its metabolites, and exogenous agents; it also plays an important role in lipid metabolism regulation (Oshida *et al*., 2015). Fatty acid synthetase (FAS) is the rate-limiting enzyme that catalyzes fatty acid synthesis. The main function of FAS is to

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catalyze the synthesis of palmitate from acetyl and malonyl CoA. Carnitine palmitoyltransferase 1A (CPT1A), which is located in the mitochondrial inner membrane, is essential for fatty acid oxidation because it catalyzes the key reaction in long-chain fatty acyl-CoA metabolism by transporting long-chain fatty acids from the cytoplasm into the mitochondria (Dyck *et al.*, 2000). Fatty acid translocase (FAT/CD36) is a membrane-bound glycoprotein that is present in platelets, mononuclear phagocytes, adipocytes, hepatocytes, and myocytes (Silverstein and Febbraio, 2009). FAT/CD36 has functions on long-chain fatty acid transport in adipocytes, muscle cells, enterocytes, and hepatocytes (Coburn *et al.*, 2000). Models wherein FAT/CD36 is specifically induced in the liver by pharmacologic approach or cDNA transduction can lead to steatosis, which can also contribute to metabolic disorders (Zhou *et al.*, 2008). Apolipoprotein AI (ApoAI) is the major protein component of high-density lipoprotein (HDL) in plasma that participates in lipid transportation. ApoAI expression can significantly reduce the liver endoplasmic reticulum stress and lipogenesis in hepatocytes (Guo *et al.*, 2017).

In conclusion, lipid metabolism disorders lead to various diseases, and the liver plays an important role in the body's lipid metabolism regulation. A considerable number of microRNAs are involved in liver lipid metabolism regulation. Therefore, this study aims to establish a rat model of lipid metabolism disorder induced by a high-fat diet, to screen differentially expressed microRNAs in the liver, and to investigate the possible effect of microRNAs in liver lipid metabolism.

Materials and Methods

Animals and grouping

All animal experiments and protocols involved in this study were approved by the Animal Care and Use Committee of the China Institute of Sport Science. Fifty 3-week old male SD rats weighing 55.36 ± 3.09 g were purchased from the Vital River Laboratory Animal Technology Co., Ltd. The Sprague-Dawley (SD) rats were raised in a temperature-controlled room (22.0 ± 0.5 °C) with natural lighting. After rats were fed with a standard chow diet for 1 week, the rats were randomly divided into two groups, namely, C (N = 10) and H groups (N = 40). C group was fed with normal rat food containing 10% of fat, H group was fed with high-fat diet containing 40% of fat for 12 weeks.

Sample collection

We selected 10 rats randomly from the H group and fed them for 12 weeks. Then we used C group rats as the control. After 12 h of fasting, the body weight, body length, perirenal fat, and epididymis fat of each rat were measured. Lee's index was calculated using the following formula: Lee's index = $(\text{body weight})^{1/3} \times 1000/\text{body length}$. The total perirenal and epididymis fat weight was used as the fat mass. The blood samples were obtained from the abdominal aorta. The serum was frozen at -80°C for chemical analysis. The right lobe of the liver was removed, rapidly frozen in liquid nitrogen, and stored at -80°C until use.

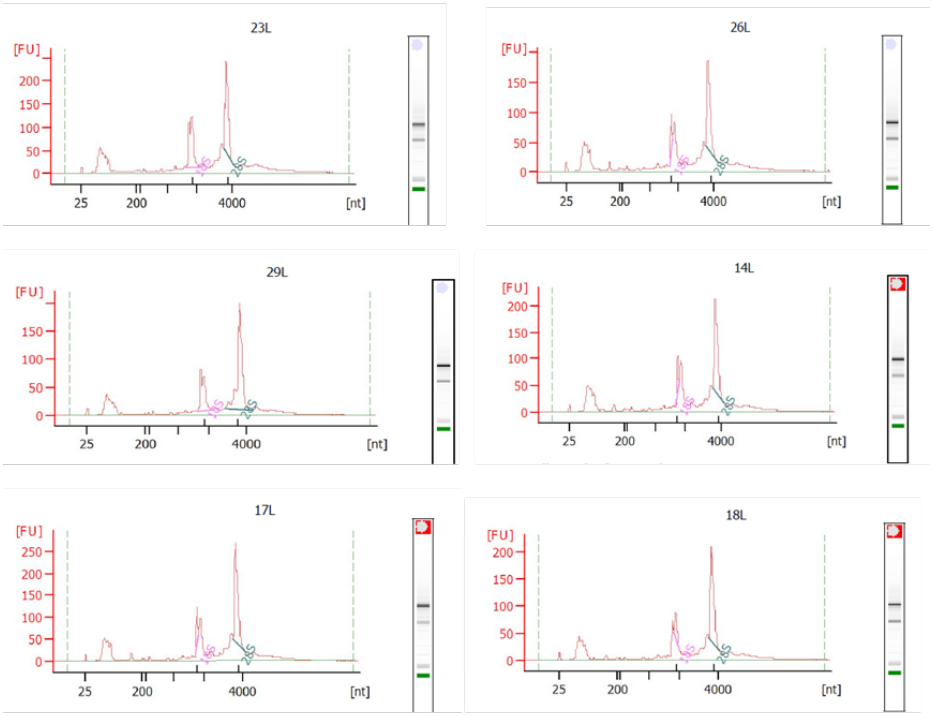

FIGURE 1. The quality of total RNA was detected by Agilent 2100 Analyzer C group (23L, 26L, 29L), H group (14L, 17L, 18L), three peaks in the map from left to right in turn were 5 s, 18 s, and 28 s.

Serum lipid concentration detection
The serum TC, TG, FFA, HDL-C, and LDL-C concentrations were detected through an automatic analyzer by using enzymatic methods (BAYER ADVIA-2400).

MicroRNA expression profile measurement by microRNA microarray
The microRNA microarray used contained 722 rat mature microRNA sequences. Three samples were randomly selected from each group (C and H groups) to extract the total RNA from rat liver. Then, the quality of total RNA was detected by an Agilent 2100 analyzer (Fig. 1). MicroRNA microarray experiments were performed by Hangzhou Lianchuan Biotechnology Co., Ltd. according to the experimental protocol, followed by the extension of the 3' end with a poly(A) tail, oligonucleotide labeling, hybridization, dyeing, fluorescence image collection, digital transformation, and Array-Pro image analysis (Lu *et al*., 2014).

Relative expression of genes related to lipid metabolism by SYBR Green real-time PCR
Eight liver samples were randomly selected from each group (C and H groups). The total RNA was extracted with TRIzol (Tiangen) according to the manufacturer's instructions. The optical absorption values of 230, 260, and 280 nm were determined by spectrophotometry to confirm RNA purity and density. The total RNA purity and integrity were determined by agarose gel electrophoresis.

cDNA was synthetized using AMV First Strand cDNA Synthesis Kit (ABI) according to the manufacturer's instructions. A total of 1 μL cDNA, 1 μL of 10 μM forward/reverse primers, 10 μL of SYBR Green qPCR Master Mix, and 7 μL of H_2O were mixed well in 0.2 mL PCR reaction tubes. Then, the tubes were placed in the LightCycler480 software setup (Roche). The reaction was incubated at 95°C for 2 min, 40 cycles at 95°C for 10 s, and then 60°C for 40 s. The primers were purchased from Sangon Biotech (Shanghai) Co., Ltd., and the sequence of primers are listed in the Tab. 1. β-actin was used as the internal parameter in the experiment. The critical threshold (ct) value was used to quantify the mRNA expression. The relative expression level was quantitated according to the following equation: $F = 2^{-\Delta\Delta Ct}$ (Lu *et al*., 2014).

Protein expression levels detected by enzyme-linked immunosorbent assay (ELISA)
Eight liver samples were randomly selected from each group (C and H groups). The Tissue or Cell Total Protein ExtractionKit (Sangon Biotech) was used to extract total protein from liver samples according to the manufacturer's instructions. The protein content of the samples was detected using Bradford Protein Assay Kit (Tiangen). A diluent was used to regulate each sample to reach the equivalent protein concentration. ELISA was used to detect the protein PPARα, CPT1A, FAS, ApoAI, and FAT/CD36 concentration according to standard procedures (Lu *et al*., 2014).

Statistical analysis
The quantitative data were expressed as mean ± standard deviation (SD), and SPSS 20.0 was used for statistical analysis. The independent-sample *t* test was used to analyze the experimental results. $p < 0.05$ indicates significant difference, and $p < 0.01$ indicates highly significant difference.

Results

High-fat diet increased rat body fat
The average body weight and body length of the H group were insignificantly different compared with those of the C group ($p > 0.05$). By contrast, Lee's index, fat mass, fat mass, and the bodyweight ratio of the H group were all significantly higher than those of the C group ($p < 0.05$, Fig. 2).

Dyslipidemia in rats caused by the high-fat diet
The serum TC, TG, HDL-C, LDL-C, and FFA levels were all significantly higher in the H group than those in the C group ($p < 0.05$, or $p < 0.01$). The HDL-C/ LDL-C ratio in the H group was significantly lower than that in the C group ($p < 0.01$, Fig. 3).

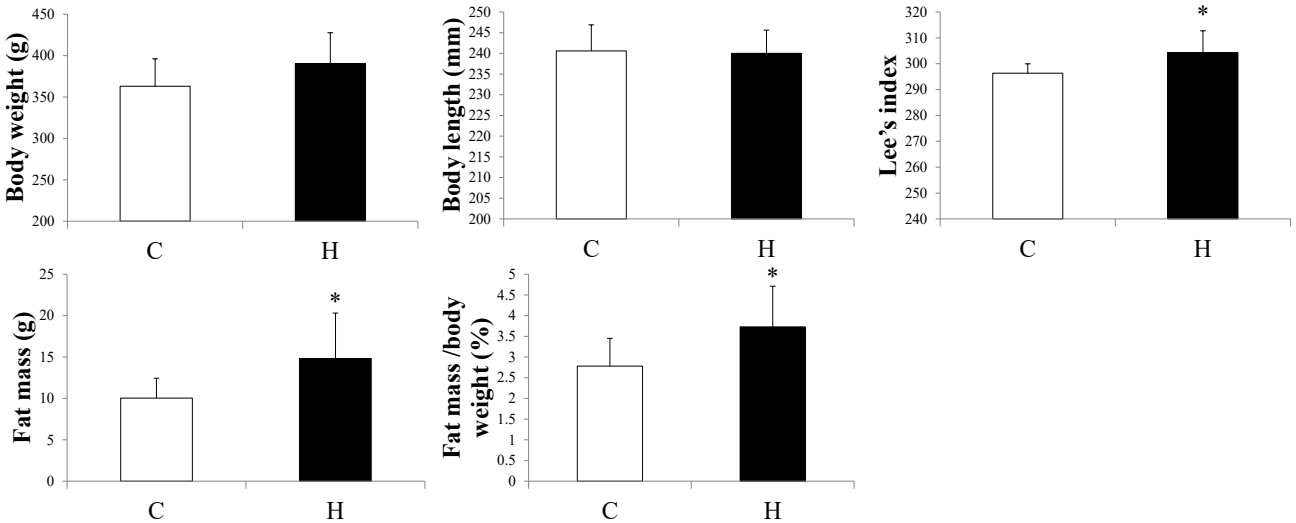

FIGURE 2. The morphological index data in the C and H group C, standard rat chow group; H, high-fat diet group. Experimental results were analyzed by an independent-sample *t* test. * $p < 0.05$, H *vs.* C.

TABLE 1

The primers of real-time PCR

Gene symbol	Refseq or Gene bank ID	Primer Sequence (5′ –3′)	Product size (bp)
β-actin	NM_031144.3	F: CGTAAAGACCTCTATGCCAACA R: CGGACTCATCGTACTCCTGCT	229
PPAR α	NM_013196.1	F: TCTGAAAGATTCGGAAACTGC R: CATGTATGACAAAAGGCGGAT	146
FAS	NM_017332.1	F: GATGAAGAGGGACCATAAAGATAAC R: CCACTTGATGTGAGGGGAGAT	157
CPT1A	NM_031559.2	F: CTGCTGTATCGTCGCACATTAG R: CGGGAAGTATTGAAGAGTCGC	110
FAT/CD36	AF072411.1	F: TGGCAAAGAATAGCAGCAAG R: ACAGTGAAGGCTCAAAGATGG	153
ApoAI	NM_012738.1	F: TCCCAGTTTGAATCCTCCAC R: CATCTCGTTTCTCAGCCAATC	168

F: forward primer; R: reverse primer. Partial primers sequences were quoted from (Lu *et al.,* 2014).

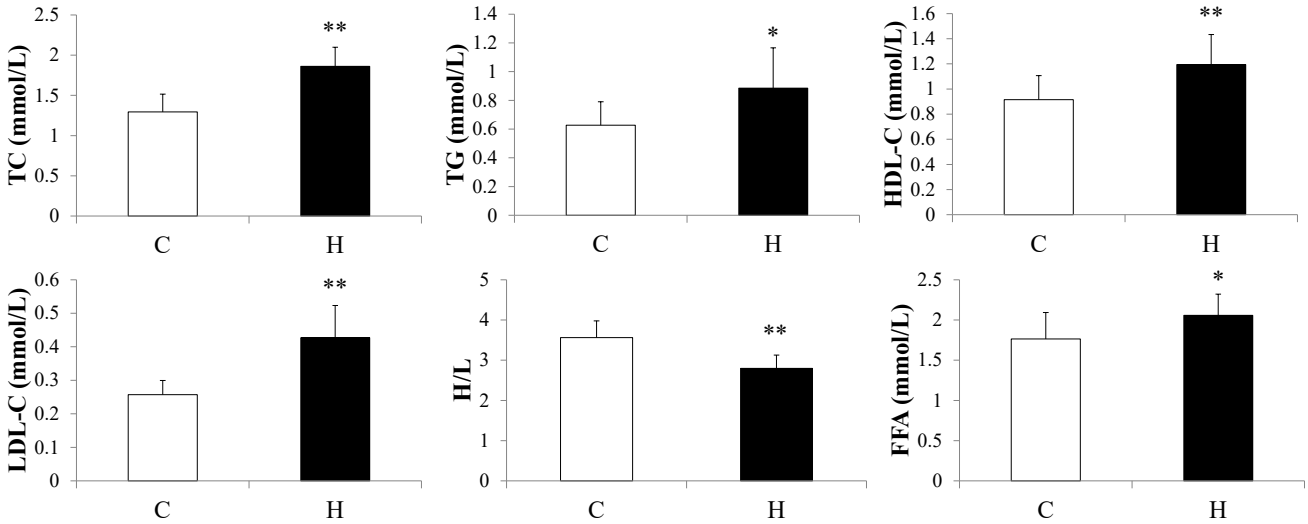

FIGURE 3. Serum lipid concentration. C, standard rat chow group; H, high-fat diet group. H/L, HDL-C/ LDL-C. Experimental results were analyzed by an independent-sample *t* test. * $p < 0.05$, H *vs.* C; ** $p < 0.01$, H *vs.* C.

High-fat diet altered partial microRNA expression levels in rat liver

MicroRNA microarray results showed that the expression levels of 10 microRNAs out of 722 mature microRNAs were significantly different between the two groups ($p < 0.05$). The miR-203a-3p and miR-6215 expression levels were lower, while those of miR-221-3p, let-7a-5p, miR-25-3p, let-7b-5p, let-7c-5p, miR-352, let-7f-5p, and miR-483-5p were higher in the H group than those in the C group (Figs. 4 and 5).

High-fat diet altered mRNA expression levels of lipid etabolism-related genes in rat liver

The PPARα and FAT/CD36 mRNA expression levels in the H group rat liver were significantly higher than those in the C group ($p < 0.01$). Meanwhile, the FAS, CPT1A, and ApoAI mRNA expression levels in the H group were all significantly lower than those in the C group ($p < 0.05$ or $p < 0.01$, Fig. 6).

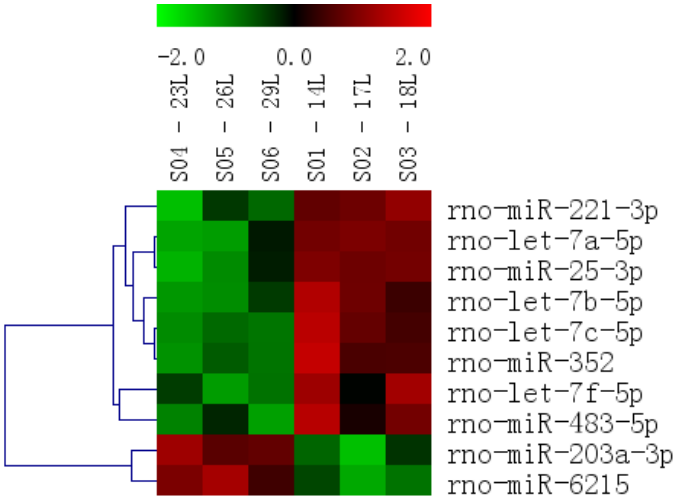

FIGURE 4. Differentially expressed microRNAs in rat liver. C group (S04-23L, S05-26L, S06-29L), H group (S01-14L, S02-17L, S03-18L).

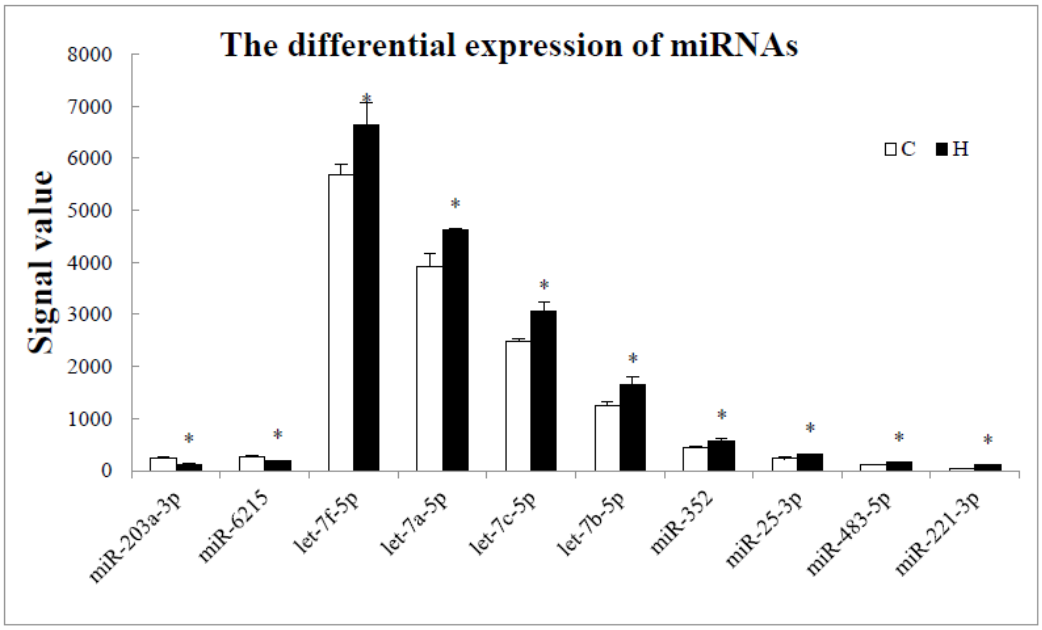

FIGURE 5. The differential expression of microRNAs. C, standard rat chow group; H, high-fat diet group. Experimental results were analyzed by an independent-sample *t* test. * $p < 0.05$, H *vs.* C.

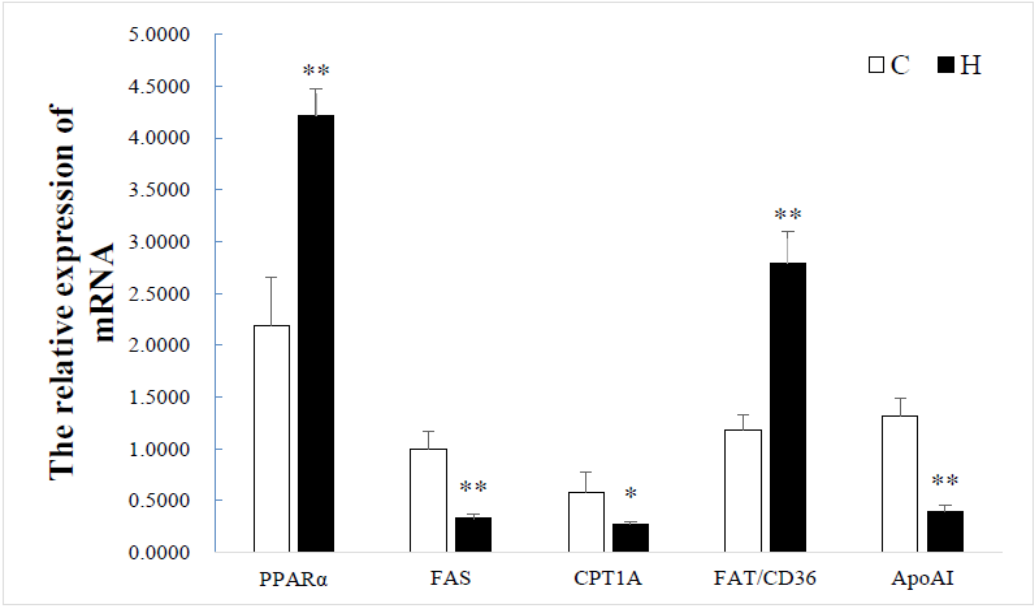

FIGURE 6. The relative expression level of mRNA in rat liver. C, standard rat chow group; H, high-fat diet group. Experimental results were analyzed by an independent-sample *t* test. * $p < 0.05$, H *vs.* C; ** $p < 0.01$, H *vs.* C.

High-fat diet altered protein expression levels of lipid metabolism-related genes in rat liver

The protein PPARα, FAS, CPT1A, FAT/CD36, and ApoAI expression levels were all significantly lower in the H group than those in the C group ($p < 0.05$ or $p < 0.01$, Fig. 7).

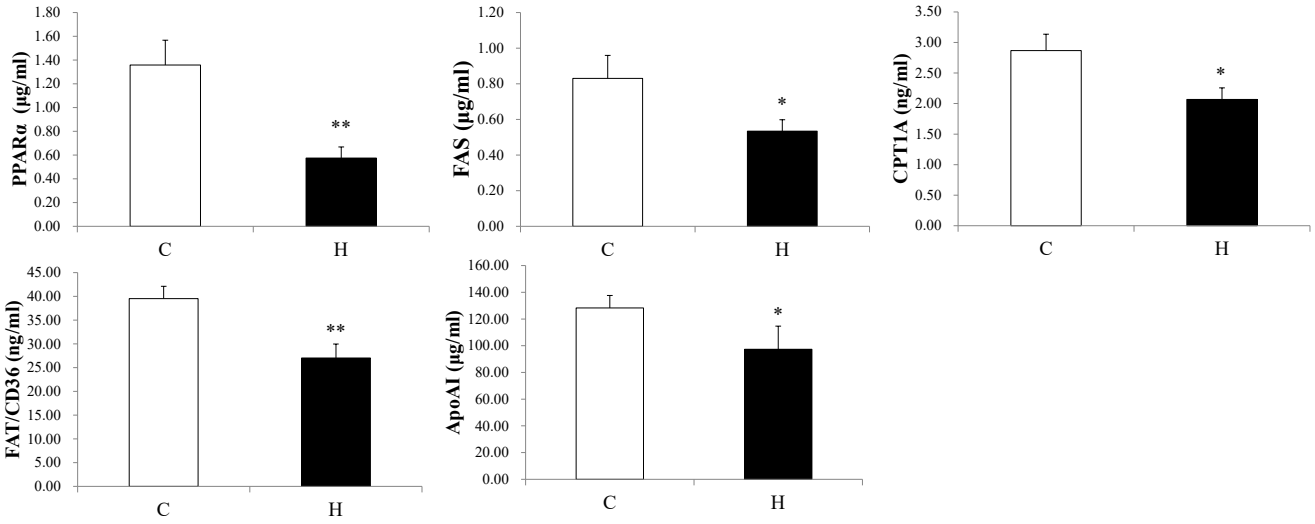

FIGURE 7. The expression level of protein in rat liver. C, standard rat chow group; H, high-fat diet group. Experimental results were analyzed by an independent-sample *t* test. * $p < 0.05$, H *vs.* C; ** $p < 0.01$, H *vs.* C.

Discussion

High-fat diet increased rat body fat, thereby causing disorder in serum lipid metabolism

High-fat diet causes the excessive intake of energy, especially excess fat, and increases the rat body weight, body fat, and serum lipid content (Sishi *et al.*, 2011; Buettner *et al.*, 2006; Erdei *et al.*, 2006; Dobrian *et al.*, 2000). However, body weight, body fat, and serum lipid concentration increase to varying degrees in different studies. In the present study, high-fat diet rats showed a significant increase in the body fat and serum lipid content, while insignificant weight gain was observed in high-fat diet group rats, which was different from the results in previous studies. The phenomenon may be attributed to the different experimental subjects, subjects' ages, fat contents, and high-fat feeding durations. Another important reason is that individuals have different susceptibility to a high-fat diet, with some becoming obese and others becoming resistant to obesity. Levin *et al.* (1997) suggested that obesity-resistant rats in a closed group accounted for approximately 30%. Therefore, although the rat body weight does not increase significantly, high-fat diet induces a significant increase in body fat, results in serum lipid metabolism disorder, and impairs liver function in rats. The rats may be invisible obesity with some degree of metabolic syndrome.

High-fat diet altered partial microRNA expression level in rat liver

MicroRNAs are important post-transcriptional regulators that are involved in the regulation of many biological processes. MicroRNAs are expected to regulate the expression of approximately 30% of the protein-encoded genes in mammals (Friedman *et al.*, 2009). A large number of microRNAs (miR-122, miR-181b, miR-185, miR-212, and miR-149) is involved in lipid metabolic processing in the liver (Wu *et al.*, 2017; Wang *et al.*, 2017; Wang *et al.*, 2014; Xiao *et al.*, 2016a; Xiao *et al.*, 2016b). However, the target genes of different microRNAs are different, and the regulation mechanisms of different microRNA are diverse.

The let-7 family regulates glucose metabolism in multiple organs. Let-7 participates in the skeletal muscle glucose metabolism of patients with type II diabetes and let-7a and let-7d overexpression in the skeletal muscle suppresses glucose uptake, oxidation, and glycogen synthesis of patients with type II diabetes (Jiang *et al.*, 2013). The global and pancreas-specific let-7 overexpression in mice results in impaired glucose tolerance and reduces glucose-induced pancreatic insulin secretion. The global knockdown of the let-7 family is enough to prevent and treat impaired glucose tolerance in mice with diet-induced obesity, at least in part, by improving insulin sensitivity in the liver and muscles. The anti-miR treatment of mice on a high-fat diet prevents ectopic fat deposition in the liver (Frost and Olson, 2011).

In the present study, the expression levels of 10 microRNAs from the liver of the C group showed significant differences compared with those of the H group. A part of the let-7 family (e.g., let-7a-5p, let-7b-5p, let-7c-5p, and let-7f-5p) showed increased expression in the H group. The research showed that the sequence of let-7 family was highly conservative (Tab. 2), thereby indicating that members of this family regulates the same target genes and play similar biological function (Frost and Olson, 2011). In this experiment, the differences in expression levels among all the microRNAs were measured, and the let-7 family (let-7a-5p, let-7b-5p, let-7c-5p, let-7f-5p) showed high values between

the C and H groups. Overexpressed let-7 family regulated glucose metabolism and increased the ectopic fat in the liver of the rat undergoing high-fat diet. Therefore, let-7 family may be an important microRNA in adjusting the hepatic carbohydrate and lipid metabolism of the rats supplemented with a high-fat diet. Thus, the regulating mechanism of let-7 family in rat liver must be studied.

TABLE 2

The sequence of rno-let-7 family

microRNA	microRNA sequence (5′ –3′)
rno-let-7a-5p	UGAGGUAGUAGGUUGUAUAGUU
rno-let-7b-5p	UGAGGUAGUAGGUUGUGUGGUU
rno-let-7c-5p	UGAGGUAGUAGGUUGUAUGGUU
rno-let-7f-5p	UGAGGUAGUAGAUUGUAUAGUU

The effect of microRNA differentially expressed in this experiment on liver lipid metabolism is unclear. We must predict the target genes of microRNA differentially expressed using bioinformatics methods (i.e., TargetScan, PicTar, and miRanda) to predict the Gene Ontology function and KEGG signal pathway annotation of the target genes and verify the function of target genes, which may be involvement in liver lipid metabolic regulation, by using a luciferase report gene. The next step of our research focused on the function of the differential microRNA expression in liver, whether the microRNA is involved in hepatic lipid metabolism regulation, and which target genes are regulated.

High-fat diet for 12 weeks changed expression level of some genes related to lipid metabolism regulation

Hepatic lipid metabolism is affected by TG and fatty acid synthesis and degradation balance. Breaking this balance leads to fatty liver degeneration. Fatty liver degeneration has many experimental animal models. Rodent model induced by high-fat diet is a useful model in studying the physiological and pathological molecular mechanisms of hepatic steatosis.

The high-fat diet changes the expression level of the genes and proteins related to liver lipid metabolism and impairs liver function. The results of gene chip in rats fed with high-fat diet showed that the majority of the differentially expressed genes in liver were involved in lipid synthesis metabolism and fatty acid oxidation (Buettner *et al.*, 2006). A high-fat diet increases PPARα mRNA expression (Burgueño *et al.*, 2013). However, another study showed that PPARα is upregulated, with nonsignificant difference (Buettner *et al.*, 2006). A high-fat diet increases the expression levels of fatty acid uptake (FAT/CD36) and synthesis-related genes (FAS) in the liver (Buettner *et al.*, 2006; Nishikawa *et al.*, 2012; Gregoire *et al.*, 2002). However, another study has also shown that the FAS expression is downregulated in the liver of mice fed with high-fat diet (Kim *et al.*, 2004). Most studies showed that a high-fat diet can upregulate lipogenic-related genes and downregulate fatty acid oxidation-related genes, thereby resulting in excessive TG accumulation in the liver, which can lead to liver damage and aggravate lipid metabolism disorders. However, opposite results have been found for the expression level of some genes (De Fourmestraux *et al.*, 2004; Patsouris *et al.*, 2006).

The results of the studies above showed that high-fat diet has varying effects on the expression of the lipid metabolism-related genes in rodent liver. The expression of genes related to lipid metabolism regulation may vary according the nature of experimental animals, high-fat diets, and different experimental protocols. The duration of a high-fat diet may be the key factor affecting gene expression. Short-term high-fat diet can increase the expression of lipogenic genes. Meanwhile, a long-term high-fat diet reduces or induces the expression level of fatty acid and cholesterol synthesis gene to return and increases or inhibits fatty acid oxidation and uptake gene expression. Rats fed with a high-fat diet for 12 weeks showed hepatic lipid accumulation due to excessive fat intake. Lipid aggregation in the liver increases lipolytic enzyme expression and reduces lipid synthase expression through feedback mechanisms of substance metabolism, thereby accelerating the lipid metabolism in the liver. Therefore, the decrease or invariability of lipid synthesis in the long-term high-fat diet animal liver may be due to the physiological adaptability of the excessive lipid intake.

In the present study, the PPARα, FAS, CPT1A, FAT/CD36, and ApoAI protein expression levels were all significantly reduced in the H group. The results showed that the level of lipid metabolism, including fatty acid synthesis, oxidation, and transport, decreased in the liver. The results may be due to the liver damage caused by lipid accumulation, thereby decreasing lipid metabolism function. The CPT1A, FAT/CD36, and ApoAI expression levels were regulated by PPARα. The results of this study showed that the CPT1A, FAT/CD36, and ApoAI protein levels decreased at the same time, thereby verifying the regulatory effect of PPARα on lipid catabolism. These results indicated that high-fat diet may inhibit fatty acid b-oxidation, reduce fatty acid transport, and decrease hepatic fatty acid catabolism by reducing the PPARα protein expression level in the rat liver. The decreased FAS expression in the liver may be affected by high-fat diet duration. The long-term high-fat diet resulted in excessive fat accumulation in the liver and caused liver fatty lesions. Out of its own protective reaction, it inhibited the FAS expression and reduced lipid synthesis and liver damage. Considering a significant increase in the serum HDL-C content, a considerable amount of cholesterol was transported into the liver, the burden of the liver was increased, and ApoAI expression was inhibited.

In summary, the long-term high-fat diet resulted in liver lipid accumulation and caused liver damage, thereby reducing hepatic involvement in lipid metabolism function. The lipid metabolism level in the liver decreased, which in turn promoted liver lipid accumulation and injury, thereby forming a vicious cycle. Therefore, the gene expression levels of lipid synthesis and degradation in rat liver are affected by high-fat diet duration, serum lipid levels, lipid content in the liver, and other internal and external factors regulated by complex regulatory networks.

Conclusions

A high-fat diet increased the SD rat body fat and serum lipid levels and changed the expression levels of 10 microRNAs in

the liver. The high-fat diet may regulate hepatic carbohydrate metabolism and increase ectopic fat accumulation through let-7 family overexpression. Meanwhile, the effect of the differentially expressed microRNAs on hepatic lipid metabolism related genes was unclear. The 12 weeks of a high-fat diet may decrease fatty acid synthesis by downregulating FAS expression and reduce fatty acid transport, b-oxidation, and fat decomposition rate by downregulating the PPARα, CPT1A, FAT/CD36, and ApoAI expression levels in rat liver.

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