The evaluation for CpG island methylation of FASN and SREBP promoter in Mongolian gerbils NAFLD model

Type
Research paper

Keywords
nonalcoholic fatty liver disease (NAFLD), Mongolian gerbil, FASN, SREBP-1, CpG island methylation

Abstract

Introduction
The methylation of CpG island in promoter and its nearby area is one of the most important way to inhibit gene expression. The aim of this study was to examine the potential relationship between the CpG island methylation for fatty acid synthase (FASN) and sterol regulatory element-binding protein 1 (SREBP-1).

Material and methods
Ten newborn pups of gerbils as NB group, 10 adult gerbils with normal diet as control group, 10 adults with high-fat diet as NAFLD group, and 10 8-month-old as aged group. Blood and liver samples were collected for serum lipid detection and histopathology. A pyrosequencing technique was employed to determine the methylation rate. Then, the transcription and expression level for FASN and SREBP-1 were verified.

Results
Serum cholesterol and triglyceride was significantly increased in NAFLD and aged group (vs. control, P<0.05). The gerbils in NAFLD and aged group also showed obvious hepatic steatosis confirmed by histological examination. Control group had the highest methylation rate for FASN and SREBP-1, which were reduced in NAFLD and aged group. Except for NB group, both the transcription and expression levels of SREBP-1 and FASN genes were presented control group > Aged group > NAFLD group. Genes of SREBP and FASN showed a trend of hypomethylation in the NAFLD gerbil model.

Conclusions
The expression of SREBP gene tended to decrease, while the expression of FASN gene tended to increase with the age and disease development. FASN and SREBP-1 methylation might be a new method to evaluate NAFLD animal model and an available target for genetic marker screening.
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**Keywords:** Nonalcoholic fatty liver disease (NAFLD); Mongolian gerbil; FASN; SREBP-1; CpG island methylation

**Outline:** The methylation rate of CpG island for FASN and SREBP-1 is a new index to evaluate NAFLD model and a available target for genetic marker screening.

**Competing interests**

The authors declare that they have no competing interests.

**Funding**

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**Ethics approval and consent to participate**

The use of animal in this study has complied with all relevant and institutional policies and was approved by the Ethics Committee for Research on Laboratory Animal Use of Zhejiang Academy of Medical Sciences.

**Availability of data and materials**

The datasets analyzed during the current study are available from the corresponding author on reasonable request.

**Patient consent for publication**

Not applicable.
**Introduction**

Nonalcoholic fatty liver disease (NAFLD) is reported to be associated with epigenetic DNA methylation and nutritional status[1]. In adult mammalian, 70 percent of DNA methylation happens in the CpG sites, while nearly 70-80 percent CpGs genomes are methylated in human[2]. DNA methylation mainly regulates gene expression through affecting the binding rates of genes and transcription factors[3]. One of the most important ways to inhibit gene expression is the dynamic methylation of CpG islands in promoter as well as its nearby regions[4, 5]. More than 1/3 genetic diseases caused by base conversion is attributed to cytidine methylation[6]. Mello VD et al found 1,292 CpG methylation among 677 genes in NASH population (non-alcoholic steatohepatitis, the second stage of NAFLD)[7]. Based on the analysis for 45,000 CpG loci, Ahrens et al observed 467 dinucleotides with methylation in NASH patients in comparison to the non-obese population[8]. Additionally, they revealed there were eight genes involved in NAFLD and metabolism disease whose CpG methylation reversed[8].

In NAFLD field, rat and mouse are the most common animals used in researches, but they have many obvious disadvantages, such as long modeling period (more than 48 weeks), large difference in lipid metabolism from human, low survival rate[9]. Our research group has been studying hyperlipidemia in Mongolian gerbil model since 2005, successfully established NAFLD model in Mongolian gerbils[10]. NAFLD development in this animal has three distinctive stages (steatosis, steatohepatitis and fibrosis), mimicking the natural pathogenesis in human. Moreover, the model could be available in short time (1-12 weeks). As these outstanding improvement, the model has received the experimental animal science and technology achievement award of China in 2016.

To study the underlying mechanism of NAFLD in Mongolian gerbils, two genes were
selected in this research, fatty acid synthase (FASN) and sterol regulatory
element-binding protein 1 (SREBP-1), which were crucial to lipid regulation and
metabolism. We cloned part promoter sequence of both genes, detected the number
and frequency of CpGs at specific locations by pyrosequencing as well as determined
the expression by quantitative real-time PCR (qPCR) and western blot (WB) analysis, in
order to investigate the potential mechanism from the perspective of epigenetic
methylation.

Materials and methods

Animal models. All the animals used in the study were male (Key Laboratory of
Experimental Animal, Zhejiang Academy of Medical Sciences, China, Certificate NO:
SCXK (Zhejiang) 2014-0001, SYXX (Zhejiang) 2014-0008]. Twenty 3-month-old gerbils
(Meriones unguiculatus) of 50-70g body weight were divided into control group (n=10)
and NAFLD model group (n=10).

In addition, ten 8-month-old gerbils weighing 80-100g were as aged group and ten
newborn gerbils were as NB group. NAFLD group was fed with high-fat diet (7% lard,
2% cholesterol, 0.5% bile salts and 10% yolk powder) while other two groups with
standard diet. Blood and liver were collected for further detection after 4-week
feeding in control and NAFLD group, while NB and aged group were harvested at the
same time. All the animals were sacrificed by anesthesia with carbon dioxide.

The use of animal in this study has complied with all relevant and institutional
policies and was approved by the Ethics Committee for Research on Laboratory
Animal Use of Zhejiang Academy of Medical Sciences.

Biochemical analysis and HE staining. Serum (except NB group) was extracted for lipid
detection including total cholesterol (TC), triglycerides (TG), high-density lipoprotein
(HDL) and low-density lipoprotein (LDL), determined by an automatic chemical
analyzer (AUTOLAB PM4000, Italy) with commercial kit (Diasys Diagnostic Systems, Co.
LTD., Shanghai, China) according to the manufacturer’s instructions. One piece of liver
tissue from right lobe was used for the histological examination by HE staining.

Capture of promoter regions in FASN and SREBP. Primers were designed according to the sequences of human, rat and mouse as well as the amplification efficiency, fragment size and specificity. Primers were designed using Primer-blast software, and primer comparison was also performed using Blast in order to reduce the non-specific amplification. Gerbil’s liver (100 mg) was made into TEN cell suspension, and then the genomic DNA was extracted with the dissolution in TE buffer solution (pH 8.0) using the magnetic bead method. Next, the purity and concentration of extracted DNA were measured, with the 260/280 ratio>1.6 indicating good quality. The reaction liquid was diluted into 100 ng/μL with TE buffer solution for the next assay at 4°C. Primers for capturing the promoter regions in FASN and SREBP were listed in Table 1. PCR Mix (15 μL) contained 12.5 μL KAPA2G Robust Mix. 1 μL each of forward and reverse primers (10 mM), 1 μL template DNA and 9.5 μL ddH2O. The thermal cycling profile for PCR was set up as follows: 94°C for 5 min, 94°C for 30 s, Tm for 30 s, 72°C for 1 min, 72°C for 5 min, followed by heat preservation at 4°C. PCR was performed on the Applied Biosystems 9700 PCR System.

Detection of CpG islands in the promoter regions of FASN and SREBP. DNA was treated with sulfite conversion for the methylation detection. Each sulfite mixture was added 800 μL RNAse-free H2O, respectively. PCR for sulfite conversion was performed in a total volume of 140 μL containing 40 μL DNA solution (concentration between 1-500 ng/μL), 85 μL sulfite mixture and 15 μL DNA protection solution. The PCR program was set up as follows: denaturation at 95°C for 5 min, renaturation at 60°C for 25 min, denaturation at 95°C for 5 min, renaturation at 60°C for 85 min, denaturation at 95°C for 5 min, and renaturation at 60°C for 175 min. Three cycles were performed between the intervals of renaturation. PCR products were kept at 20°C overnight. Next, DNA was purified with disulphonic acid solution using the method of adsorption column, followed by elution in elution buffer. The purified DNA was used directly in the next
methylation-specific real-time PCR (MSP) analysis.

MSP was performed in a total volume of 50 μL containing 10 μL 5×buffer GC (KAPA), 1 μL dNTP (10 mM/each), 1 μL each of upstream and downstream primers (50 pM/μL), 2 μL template DNA and 0.2 μL Taq (5U/μL). The thermal cycling profile for MSP was set up as follows: 40 cycles of 95°C for 3 min, 94°C for 30 s, 52°C for 30 s, 72°C for 1 min, and a final 7 min at 72°C. Primers for MSP of FASN1 and SREBP1 were listed in Table 2.

Next, mixed beads together with PCR products formed a suspension, immersing into 70% ethanol, 0.2M NaOH and flushing buffer to cool to room temperature. Next, the mixture denatured at 85°C for 2 min, and then the universal primer (cool to room temperature) for sequencing (S primer) hybridized with the template DNA. Substrate mixture, enzyme mixture and 4 dNTP (QIAGEN) was added successively in the reagent chamber, of which the dosages were calculated according to the information of sequence design by PyroMark Assay Design software version 2.0. Finally, the reagent chamber together with 96-hole reacting plate was put into the Pyrosequencing detector (PyroMark Q96 ID, QIAGEN) for the reaction. The methylation status of each site was automatically analyzed using Pyro Q-CpG software.

**FASN and SREBP-1 Expression.** Quantitative real-time PCR (qPCR) was performed on the samples derived from liver tissue. For qPCR analysis, cDNA was synthesized from total RNA by reverse transcription with RT-reagent Kit and DNA Eraser (TaKaRa Biotech, Kyoto, Japan). Primer synthesis of mRNA was designed using Primer 5.0 and Oli906.0, and β-actin was used as internal control. qPCR reaction was performed in a total volume of 20 μL containing 10 μL 2×fluorescence qPCR Mix, 1 μL each of upstream and downstream primers (10 μM), 1 μL template DNA and 7 μL ddH2O. The thermal cycling profile for qPCR was set up as follows: 40 cycles of pre-denaturation at 94°C for 1 min, denaturation at 94°C for 10 s, annealing at 59°C for 10 s, followed by extension at 72°C for 10 s. The results of qPCR were determined with the 2-ΔΔCt method. Primers for qPCR were listed in Table 3.

For Western blot, protein lysates from liver were quantified by the BCA kit
(Tiangen Biotech Co. Ltd., Beijing, China). 30μg of Proteins extracts was separated on 12% SDS-PAGE gels and transferred onto the PVDF membranes. After blocked with 5% non-fat milk, the membranes were incubated with primary antibody of FASN and SREBP-1 (1:500 dilution). Following secondary antibody incubation and PBS washing, the bands were visualized by X-ray exposure. Quantification was performed using the ImageJ software.

Statistical analysis. When applicable, results were presented as means ±standard deviation (SD). The Tamhane's T2 method was used for the comparison of serum lipid level and paired t-test for gene expression. Correlation between the expressions of FASN and SREBP and methylation rate were analyzed by Pearson's correlation test. All statistical analyses were performed using the SPSS software (version 13.0, SPSS Inc. Chicago, IL, USA). A two-sided p<0.05 was considered to be statistically significant.

Results

Hepatic morphology. In the gerbil NB group, the structure of hepatic lobule was clear, the hepatic cord was neatly arranged, and the hematopoietic cells of extramedullary hematopoietic foci were observed in the hepatic sinusoid, in which a large number of mononuclear cells and red cells were found (Fig. 1A). In the control group, no obvious fat deposition in the abdominal wall of the gerbils was observed; the liver was not obviously changed, which appeared red with sharp edges, clear lobules and plate-like arranged hepatic cords (Fig. 1B). In the NAFLD and aged group, the liver appeared yellow with blunt edges and greasy feelings; parts of the gerbils were observed swollen in the spleen, in which the HE staining showed obvious hepatic steatosis, and the NAFLD group is more serious pathomorphology than that of the aged group, with hepatocyte necrosis and shedding widely distributed (Fig. 1C, 1D).

Biochemical index. The serum levels of TC, TG, HLD and LDL are given in Table 4, the serum TC level of the gerbil NAFLD group multiplied rapidly to 5.3-fold higher
compared to the gerbil control and aged group, and the serum levels of HDL (6.5-fold) and LDL (4.3-fold) were also significantly increased ($p<0.01$). The serum TG level of the gerbil aged group significantly increased compared to the gerbil control and NAFLD group (5-fold, $p<0.01$). It indicates to some extent that the two types of fatty liver models are in different stages of NAFLD, or different types hyperlipidemia.

*Sequences in the promoter regions of FASN and SREBP.* Two long fragments in the promoter regions of FASN and SREBP were captured, respectively. The sequences of the promoter regions of FASN and SREBP genes referred to the attached sequence data.

*Number of CpG island in the liver of gerbil.* The methylation levels showed dynamic differences in different regions of the same promoter region of genes. For the FASN gene, the methylation rate was highest in the gerbil control group, followed by the gerbil NAFLD and aged group ($p<0.05$). For the SREBP gene, the methylation rate was also highest in the gerbil control group, yet followed by the gerbil aged and NAFLD group ($p<0.05$). The results above indicated that methylation levels of FASN and SREBP gradually decreased in NAFLD with the supplement of high-fat diet (except the gerbil NB group), which was closely associated with hyperlipidemia and NAFLD(Fig. 2, Fig.3).

*Transcription and expression levels of FASN and SREBP.* In the transcription level, the FASN gene was highest in the gerbil NB group, followed by the gerbil aged, NAFLD and control group. Yet, for the SREBP gene, the transcription level was highest in the gerbil control group, followed by the gerbil NB, aged and NAFLD group (Fig. 4). The expression levels of FASN and SREBP genes were presented in Fig. 5. For the FASN gene, the expression level was highest in the gerbil aged group, followed by the gerbil NAFLD, NB and control group. Yet, for the SREBP gene, the expression level was highest in the gerbil control group, followed by the gerbil aged, NB and NAFLD group.

The correlation between gene expression and methylation rate was computed. For the FASN gene, the methylation rate was negatively correlated with the expression
level in the gerbil NB ($r = -0.78$) and control group ($r = -0.61$), while this trend weakened in the gerbil NAFLD ($r = -0.11$) and aged group ($r = 0.04$), which was consistent with the results of WB analysis. For the SREBP gene, positive and negative correlations between methylation rate and expression level was found in the gerbil NB ($r = 0.22$) and NAFLD group ($r = -0.29$), respectively. However, based on high methylation rates, the expression levels in the gerbil aged ($r = 0.05$) and control group ($r = -0.07$) tended to be weakened with the increase of methylation rates.

**Discussion**

The methyl of DNA methylation is mainly derived from the methionine, folic acid and choline in food. High-fat diet feeding (methyl-donor-deficient diet, choline and L-amino acids) for animals can affect the gene expressions through the change of DNA methylation. Methylene tetrahydrofolate reductase (MTHFR) is a key enzyme in the metabolic process of folic acid, which involves in the methionine metabolic cycle and DNA methylation through transferring the 5, 10- methyltetrahydrofolate (MTHF) into 5-MTHF[11]. Folic acid deficiency can interfere with the metabolism of methionine, resulting in a decreased methyl donor in body; therefore, hypomethylation occurs in the genome of liver, promoting the liver injury[12]. The above is the main reason for inducing the fat deposition in the liver of animals[13], and also the important theory for establishing the high-fat diet-induced animal model. In the study conducted by Wang et al, progress in pathological changes of NAFLD (fatty liver-fibrosis-NASH) were found to be closely associated with the alteration of genome methylation in the liver, which was related to the gradually decreased methyl donors due to the disorder of methionine cycle[14]. Metabolic syndrome (including hyperlipidemia) is often associated with the balance disorder between methyl supplement and methyl consumption[15]. In addition, the change of DNA methylation is not only accumulated as time proceeds, but also is influenced by eating habit, body weight, aging and environment[16,17]. Epigenomic studies of mouse models indicated that high-fat diet and exercise can change the lipid metabolism pathway and process of muscle
development, of which exercise can significantly change the gene hypermethylation caused by high-fat diet[18]. Fujiki et al found that the DNA methylation of specific gene (PPARy) could change the expression of specific genes in adipocytes[19], while Kovalenko et al found that the drug-induced (pyrazinamide) liver injury was related to the decrease of overall DNA methylation level in the liver genome, with a dose-dependent effect[20]. Therefore, it could be speculated that the fatty liver of gerbils with NAFLD (induced model) was not only related to the changes of DNA methylation caused by high-fat diet, but also related to the time cumulative effect of DNA methylation and aging (8-month-old gerbil’s model).

The methylation levels showed dynamic differences in the different regions of the same promoter region of gene. For the FASN gene, the number of CPG islands selected from the two segments FASN-6R1-1S and FASN-6R1-2S, showed a different distribution in each group. The methylation rate change dynamically of these groups were affected by diet, age and NAFLD progression. Hypermethylation in the gerbil control group compared to the gerbil NAFLD and aged group \((p<0.05)\); for the SREBP gene, the methylation rate was also higher in the gerbil control group compared to the gerbil aged and NAFLD group \((p<0.05)\). The results above indicated that methylation levels of FASN and SREBP gene gradually decreased in NAFLD with the addition of high-fat diet except the newborn gerbils (the overall DNA methylation levels in the liver genome are relatively high, inhibiting the expressions of lipogenic genes, which may be closely related to the reprogramming and environmental factors at the embryonic stage)[21,22].

SREBP plays an important role in the metabolism of lipid and protein in the liver cells, which is a key molecule regulating the lipid metabolism and expressions of genes related to the synthesis of fatty acid and TG[23]. In this study, the transcription level of SREBP gene was higher in the gerbil control group compared to the gerbil aged and NAFLD group (except the gerbil NB group), which was highly consistent with the gene expression levels. The results above indicated that the gene expression levels tended
to decrease with the formation of NAFLD, which seemed to be inconsistent with the function of lipogenic genes. However, study conducted in goose found that high-fat diet could reduce the expression level of SREBP mRNA[25][24], with a significant negative correlation between the expression levels of SREBP gene in liver tissues and liver weight index (P<0.01)[26][25], which may be due to the excessive deposition of TG in the liver resulting in an adaptive feedback of the goose body, thus inhibiting the abundance of SREBP and reducing the expressions of genes related to the lipid synthesis[26].

FAS is a key enzyme in the fatty acid synthesis, which has binding regions SRE (sterol regulatory elements) of SREBP-1C and ChREBP (carbohydrate responsive-element binding protein) in the promoter region. So the dynamic changes of SREBP can regulate the expression of FASN. And insulin can induce the tri-dimensional hypermethylation of H3K4 histone and super acetylation of H3 and H4 of FASN to regulate the expression of FASN gene, and can also accelerate the expression of FASN gene by enhancing the combination of SREBP-1C and ChREBP[27]. In this study, the transcription and expression levels of FASN gene were both higher in the gerbil aged group compared to the gerbil NAFLD and control group, indicating that the expression levels tended to increase with the formation of NAFLD, which was consistent with the pathogenesis of NAFLD including the increase in synthesis of fatty acid and cholesterol, abnormal transport of TC and cholesterol, and inhibition of fatty acid oxidation decomposition in liver[28].

In view of the susceptibility of Mongolian gerbil to high-fat diet and differences in blood lipids between the age groups as well as the combination of results in present study, we concluded that the liver genomic DNA of gerbils was influenced by high-fat diet and age, which may be different in methylation level on multiple gene expression or signal transduction pathways. The present study provided a basis for the future research on NAFLD in gerbils.

Acknowledgements
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Figure legend

Fig. 1 Pathology of liver in each group (HE staining ×20). Fig 1 showed the histopathological modification among each treatment. In NB group, the hepatic structure was neatly arranged, and the hematopoietic cells of extramedullary hematopoietic foci were observed in the hepatic sinusoid, in which a large number of mononuclear cells and red cells were found (Fig. 1A). In control, no obvious fat deposition in the abdominal wall of the gerbils was observed; the liver was not obviously changed, which appeared red with sharp edges, clear lobules and plate-like arranged hepatic cords (Fig. 1B). In the NAFLD and aged group, the liver appeared yellow with blunt edges and greasy feelings; parts of the gerbils were observed swollen in the spleen, in which the HE staining showed obvious hepatic steatosis (Fig. 1C, 1D).

Fig. 2 Detection result of CpG island in the promoter region of FASN (Part) and SREBP(part). The CpG sites were measured using pyrosequencing, and the “methylation%” was the average of “%” of each CpG site measured. The figure presented the results of methylation rate of sample A4, A5, E1, E2.

Fig. 3 Methylation rate in the promoter regions of FASN and SREBP. Fig. 3A presented the high methylation rate region of FASN and SREBP gene of each treatment. Fig. 3B showed the low methylation rate region of the FASN gene and SREBP gene of each treatment. The letters “A / B/C” above each column indicated significant difference (p<0.01).

Fig. 4 Transcription levels of SREBP and FASN (qPCR). The transcriptional level of FASN gene for newborn, aged, NAFLD, and normal gerbils was graphed in Fig. 4A. The transcriptional level of SREBP gene for newborn, aged, NAFLD, and normal gerbils was presented in Fig. 4B.
Fig. 5 Expression levels of SREBP and FASN (Western blot analysis). Fig. 5A showed the western blot results of FASN and SREBP genes for each treatment. Fig. 5B outlined the average grayvalue of each treatment compared with β-actin.
For reviewer1:
Review 1:
The paper has been amended in accordance with reviewers' suggestions. I believe it should be accepted for publication.
Thank you for good advices to help me improve my manuscripts.

For reviewer2
Review 2:
During revision, the authors addressed most of the comments of the reviewer in their answer to the reviewers. In the manuscript, they changed a table to a figure, changed several passages within the text and added new references. Overall, the findings of the study are still very confusing and difficult to combine in one picture. One would expect that NAFLD favors either the highest or the lowest expression level, methylation level or protein level and thereby is the opposite of controls. In New Borns, one would expect either similar levels as in controls of even “better” levels. From the results presented in this study, it seems impossible to define a “rule” or a common denominator for the gene expression or degree of methylation or protein expression either during aging or during certain disease conditions. Unfortunately, the responses to the reviewers were not inserted in the manuscript, which would improve understanding of several issues. In addition, in the revised version, there are still several issues which make it difficult to follow the outline of the study.
Answer:
After careful review of the reviewer's comments, we modified the conclusion section of the abstract: increased the effect of age and NAFLD disease progression, SREBP gene expression tended to decline, and FASN gene expression tended to increase.
In the results of the paper, a detailed description of the 8-month-old model and the 3-month-old model was added to the liver morphology description to show that the two groups are at different stages of NAFLD, and the serum biochemical indicators were also added to the comparison of the two groups analysis.
The text of this article has been further modified and polished.

Lines 25/26 still do not correspond to lines 21/22.

Answer:
In this study, 8-month-old gerbils (aged group) were susceptible model to NAFLD with a normal diet, while 3-month-old gerbils (NAFLD group) were induced by high-fat diets in young gerbils. The 3-month-old model group is more serious pathomorphology than that of the 8-month-old model group, with hepatocyte necrosis and shedding widely distributed. From the serum biochemical analysis, the TC of the three-month-old model group was more than 5.3-fold that of the aged group, the HDL was more than 6.5-fold that of the aged group, and More than 4.3-fold in the LDL, while TG is less than 1/5 of the aged group (P<0.01), which indicates to some extent that the two types of NAFLD fatty liver models are in different stages of NAFLD, or different types hyperlipidemia. Combining our results of the methylation rates of the two genes, the expression levels of the FASN is the control> 3 months old model group> senile group; and SREBP is the control> old age> 3 month old model group. (referred to Liu et al., A new model:
genetic characterizations of nonalcoholic fatty liver disease in Mongolian gerbil. Hepatolology International. 2017, 11 (Suppl 1): S1-S1093, DOI 10.1007/s12072-016-9783-9. From the perspective of age and NAFLD, compared with the control (normal) group, the expression of SREBP tends to decrease, while the expression of FASN tends to increase. In accordance with line 21-22, the text in lines 25-26 was modified as follows: The expression of SREBP gene tended to decrease, while the expression of FASN gene tended to increase with the age and disease development.

Line 36 and lines 285-288: These lines do not correspond to each other.
Answer: The missing funding information in line 36 was added, which was in correspondent with the information in line 285-288.

Line 178: Why do the authors not use Arabian numbers for the table within the text, as presented with the tables?
Answer: The text in line 179 (might be somewhat different under different software in line) “table IV” was replaced with “table 4”

Table 4: A/B both indicate a significant difference with p<0.01. Then why using different letters? In which way is A different from B? Referred to classic statistics, one method to labeled the difference of the multiple comparison is labeled with different character. In this labeled method:
1) First, order the average of each treatment from large to small;
2) Then mark the letter “a” after the maximum treatment, and compare the average with each of the following treatment in order, where the difference is not significantly marked with the same letter (there “a”), until a certain treatment with a significant difference marked with the letter “b”;
3) Take the treatment number marked with the letter “b” as the standard, and compare with the treatment larger than it, and add the letter “b” if the difference is not significant until it is significant; thus some treatments may be labeled with “ab”, presents these treatments show no significant with treatments labeled with “a”, also show no significant difference with treatments labeled with “b”.
4) Then take the maximum group marked with the letter “b” as a standard. Compared with the smaller groups below, where the difference is not significant, continue to mark the letter “b” until a certain average that is significantly different from it is marked with “c”;
Repeat this process until the smallest average is marked and compared.
5) In this way, those who have the same letter between the averages are not significantly different, and those who do not have the same letter are significantly different.
6) Significant level $\alpha = 0.05$ is represented by lowercase Latin letters, and significant level $\alpha = 0.01$ is represented by uppercase Latin letters.
Based on these method, treatment labeled with “A” is different with treatment labeled with “B”.

Lines 183-187. “The promotor regions were as follows” – as follows what? To what do the color
comments refer to?
Answer: Sorry  This may be an oversight. In the first edition, due to the limitation of the manuscript size, it was decided to place the sequence in additional materials, which may have been forgotten to be added later. It now appears in the text by referring to the attachment, with the following modifications:

“The sequences were as follows (bases in different color, frame and background positions were possible methylation locations or transcription factor binding areas, which were used as reference sites for primer design of methylation sequencing).”
Was modified as “The sequences of the promoter regions of FASN and SREBP genes referred to the attached sequence data”

Lines 196-203: These lines contain two different statements about FASN gene expression in the different groups. Which one is correct?
Answer: referred to the manuscripts,

Transcription and expression levels of FASN and SREBP.  For the FASN gene, the transcription level was highest in the gerbil NB group, followed by the gerbil aged, NAFLD and control group. Yet, for the SREBP gene, the transcription level was highest in the gerbil control group, followed by the gerbil NB, aged and NAFLD group (Fig. 4).

The expression levels of FASN and SREBP genes was presented in Fig. 5. For the FASN gene, the expression level was highest in the gerbil aged group, followed by the gerbil NAFLD, NB and control group. Yet, for the SREBP gene, the expression level was highest in the gerbil control group, followed by the gerbil aged, NB and NAFLD group.

We attempted to present the transcription and expression levels respectively. The first paragraph was presented the transcription level, the second paragraph was listed the expression level, respectively. I tried to modify it this two paragraphs as following:

Transcription and expression levels of FASN and SREBP.  In the transcription level, the FASN gene was highest in the gerbil NB group, followed by the gerbil aged, NAFLD and control group. Yet, for the SREBP gene, the transcription level was highest in the gerbil control group, followed by the gerbil NB, aged and NAFLD group (Fig. 4). The expression levels of FASN and SREBP genes was presented in Fig. 5. For the FASN gene, the expression level was highest in the gerbil aged group,
followed by the gerbil NAFLD, NB and control group. Yet, for the SREBP gene, the expression level was highest in the gerbil control group, followed by the gerbil aged, NB and NAFLD group.

Lines 204-211. If the authors do not wish to show the results of a correlation in a table or in plots, as explained in their answers to the reviewers, they should put this information in the manuscript. However, this would certainly lessen the strength of their data.

Answer:
The correlation index was attached to the text. The text of related part was modified as follows:

The correlation between gene expression and methylation rate was computed. For the FASN gene, the methylation rate was negatively correlated with the expression level in the gerbil NB (r=-0.78) and control group (r=-0.61), while this trend weakened in the gerbil NAFLD (r=-0.11) and aged group (r=0.04), which was consistent with the results of WB analysis. For the SREBP gene, positive and negative correlations between methylation rate and expression level was found in the gerbil NB (r=0.22) and NAFLD group (r=-0.29), respectively. However, based on high methylation rates, the expression levels in the gerbil aged (r=0.05) and control group (r=-0.07) tended to be weakened with the increase of methylation rates.

Figure legend 3: “A / B/C indicated significant difference (p<0.01)” – then how are A, B, C different? Why using different letters?

Why are there two panels in figure 3, how do they differ? Please give more details in the figure legends of figures 2-5.

Answer:
The first question: “A / B/C indicated significant difference (p<0.01)” – then how are A, B, C different? Why using different letters? It has been explained as above. I only want to present the difference between multiple treatments. The main points are presented as following:

Referred to classic statistics, one method to labeled the difference of the multiple comparison is labeled with different character. In this labeled method:
1) First, order the average of each treatment from large to small;
2) Then mark the letter “a” after the maximum treatment, and compare the average with each of the following treatment in order, where the difference is not significantly marked with the same letter (there “a”), until a certain treatment with a significant difference marked with the letter “b”;

...
3) Take the treatment number marked with the letter “b” as the standard, and compare with the treatment larger than it, and add the letter “b” if the difference is not significant until it is significant; thus some treatments may be labeled with “ab”, presents these treatments show no significant with treatments labeled with “a”, also show no significant difference with treatments labeled with “b”.

4) Then take the maximum group marked with the letter “b” as a standard. Compared with the smaller groups below, where the difference is not significant, continue to mark the letter “b” until a certain average that is significantly different from it is marked with “c”;

Repeat this process until the smallest average is marked and compared.

5) In this way, those who have the same letter between the averages are not significantly different, and those who do not have the same letter are significantly different.

6) Significant level \( \alpha = 0.05 \) is represented by lowercase Latin letters, and significant level \( \alpha = 0.01 \) is represented by uppercase Latin letters.

The second question: Why are there two panels in figure 3, how do they differ?

The results of the methylation rates are collected from a high methylation rate region and a low methylation rate region for each gene, respectively. If it was arranged by the same gene, the high methylation rate and low methylation rate has too high different to show in the same level. So we arrange the results by the high methylation rate and low methylation rate into two panels.

The third question: The legends of fig2 to 5 were modified.

Fig. 2 Detection result of CpG island in the promoter region of FASN (Part) and SREBP(part). The CpG sites were measured using pyrosequencing, and the “methylation%” was the average of “%” of each CpG site measured. The figure presented the results of methylation rate of sample A4, A5, E1, E2.

Fig. 3 Methylation rate in the promoter regions of FASN and SREBP. A presented the high methylation rate region of FASN and SREBP gene of each treatment. B showed the low methylation rate region of the FASN gene and SREBP gene of each treatment. The letters “A / B/C” above each column indicated significant difference \( (p<0.01) \)

Fig. 4 Transcription levels of SREBP and FASN (qPCR). The transcriptional level of FASN gene for newborn, aged, NAFLD, and normal gerbils was graphed in Fig. 4A. The transcriptional level of SREBP gene for newborn, aged, NAFLD, and normal gerbils was presented in Fig. 4B

Fig. 5 Expression levels of SREBP and FASN (Western blot analysis). Fig. 5A showed the western blot results of FASN and SREBP genes for each treatment. Fig. 5B outlined the average grayvalue of each treatment compared with \( \beta \)-actin.

Figure 5 is not mentioned within the results section.

Answer:

The Fig. 5 was mentioned in the text “the transcription level was highest in the gerbil control group, followed by the gerbil NB, aged and NAFLD group (Fig. 4). The expression levels of FASN and SREBP genes was presented in Fig. 5.” (near line 200-205)

Lines 242/243: the authors state that “The methylation levels showed dynamic differences in the
different regions of the same promoter region of gene”, as they presumably present in figure 3 as well. As there are different dynamics in different regions, how then can the authors combine these differences to make a statement about the methylation rate for the FASN gene?

Answer:

1) According to our tests, the number of CPG islands selected from the two segments of the promoter region of the FASN gene, FASN-6R1-1S and FASN-6R1-2S, showed a different distribution in each group of animals. These groups were affected by diet, age and NAFLD progression that make the methylation rate of FASN gene change dynamically.

2) SREBP-1 can maintain lipids dynamic equilibrium by regulating the expression of enzymes required for synthesis of endogenous cholesterol, fatty acids, triglycerides and phospholipids (Guan Y. Aquat Toxicol. 2019 Feb;207:179-186. doi: 10.1016/j.aquatox.2018.12.011. Epub 2018 Dec 14.)

3) SREBP can bind to the SRE sequences flanking FASN, so the dynamic changes of SREBP can regulate the expression of FASN, and the same from the results of our detection of the SREBP gene promoter region.

The text in discussion part was modified as follows:

1) the number of CPG islands selected from the two segments FASN-6R1-1S and FASN-6R1-2S, showed a different distribution in each group. The methylation rate change dynamically of these groups were affected by diet, age and NAFLD progression. Hypermethylation...(line 251–254)

2) So the dynamic changes of SREBP can regulate the expression of FASN. And...(line279–280)

The authors should check the manuscript for language issues.

Answer:

we have checked the language carefully and corrected several errors(with modified label ).
The promoter region sequence of SREBP and FASN are listed as follows.

Bases in different color, frame and background positions were possible methylation locations or transcription factor binding areas, which were used as reference sites for primer design of methylation sequencing.

SREBP1-s1(1748bp)

SREBP1-s2(1770bp)
CTCTCTGCCAGTGGTGAGACTGACAGGATTAAGACAGGATCAGAAGTTCCAGCCTCTTGCTCCTCTGCAGGTCTCCTCCTCCGGAGGCCCACTTCCAGGCTCCTAGGCTGAGAGGC
TTCCGCTGAGGCCCCCGGGGCCTCGTGCGCACCCAGACCAGCATCTGGCCTCTCCGGGCTGGCGCCGGAGGACCACCGCGGCCTCCTGCGCCTGCCAAAGCCTCCCAGCCGGGAGCCCG
GAGGACCACCGCGGCCTCCTGCGCCTGCCAAAGCCTCCCAGCCGGGAGCCCG

FASN–F6R1–s1 (920bp)
GCACTTCCCAATGTCGCCGTATCACCAACCCACTCCTCCATGGCTATTTCTCTGCAGGAGGATGGCCATGAGGACCAAACAGCGATACGGCAGGAGCTGAGGCTGGCCAGCC
GAGGACCACCGCGGCCTCCTGCGCCTGCCAAAGCCTCCCAGCCGGGAGCCCG

FASN–F6R1–s2 (920bp)
TAGATTTAAGCGAAATATTACCTCAGCTCGGCTGATACACCACTCCTCCATGGGCTATTTCTCTGCAGGAGGATGGCCATGAGGACCAAACAGCGATACGGCAGGAGCTGAGGCTGGCCAGCC
GAGGACCACCGCGGCCTCCTGCGCCTGCCAAAGCCTCCCAGCCGGGAGCCCG
| Primer name | Primer sequence (5' to 3') | TM | Length |
|-------------|-----------------------------|----|--------|
| SREBP1-R1   | GGACACGCACCTTCGTGT          | 62 | 1.8K   |
| SREBP1-F1   | CCTGACCCTCCCTGGTGAC         | 64.2|       |
| SREBP1-R2   | CCTGTGACTTAGGAACAGGTAAAGC   | 61.7| 1.8K   |
| SREBP1-F2   | GACTGCACAATGCTGAGCCT        | 61.6|       |
| FASN-R1     | TGAGGTTGGCCCAAGAACTC        | 61 | 1.0K   |
| FASN-F1     | GGCCTCAGCGGAAGTCAT          | 61 |       |
| FASN-R2     | TTGCCCAGGGCAGTTCAG          | 63 | 1.0K   |
| FASN-F2     | CGGCCTCGGTGTCCAATT          | 64.4|       |
| FASN-R3     | CCACATCGAWACCAATGGAC        | 62.4| 1.0K   |
| FASN-F3     | CAGTGTTCCCTATCCTGCCTACT    | 61 |       |
| FASN-F4     | CCACGATGACCGGTAGTAAC        | 58 | 1.2K   |
| FASN-R4     | CGCTGGAGCACAAGGAAC          | 60.6|       |
| FASN-F5     | GGACAGAGATGAGGGCGTC         | 60.8| 1.2K   |
| FASN-R5     | AGCCGTAGTCCAAGGAGAAG        | 58.5|       |
| FASN-F6     | GKCTGGGYGCCTCGTGGAAT        | 62.6| 1.5K   |
| FASN-R6     | AGCCGTAGTCCAAGGAGAAG        | 58.5|       |

F and R indicated the forward and reverse primer sequence
Table 2. Primer sequences for the methylation-specific PCR of FASN and SREBP

| Primer name       | Primer sequence (5' to 3')                              | 5' modification |
|-------------------|--------------------------------------------------------|----------------|
| 1.FASN-F6R1-1F    | AGTAAGGATTTAGAAGGTTTTGG                              |               |
| 1.FASN-F6R1-1R    | CTTAAAAACCCACCTCTTTACA                                | 5'-Biotin     |
| 1.FASN-F6R1-1S    | AGAAAGGTTTTAGTGGA                                     |               |
| 2.FASN-F6R1-2F    | GGTTTTTAAGGTATTTGTAGGTA                               |               |
| 2.FASN-F6R1-2R    | CCAACCCCAAAAAACCAAATACTA TCT                         | 5'-Biotin     |
| 2.FASN-F6R1-2S    | TTAGGGTAGGTAGGT                                      |               |
| 3.SREBP1-1F       | TTGGGAGGTGAGGAAGTTATTT                               |               |
| 3.SREBP1-1R       | AAAACATTCTAACCACAAATATCAC                            | 5'-Biotin     |
| 3.SREBP1-1S       | GAGGTGAGGAAAGTTATTA                                  |               |
| 4.SREBP1-2F       | AGAGTAAAGAGTAGGTGGGTTTTTAT                           |               |
| 4.SREBP1-2R       | AATTCAAAACCCTTATCTAACCTAA TTT                        | 5'-Biotin     |
| 4.SREBP1-2S       | GGTTTAGGTGTTGGTA                                     |               |
F and R indicated the forward and reverse primer sequence, and S indicated the primer sequence of pyrosequencing.

**Table 3. Primer sequences for qPCR of SREBP-1 and FASN**

| Primer       | Sequence (5' to 3')          | Size   |
|--------------|------------------------------|--------|
| β-actin-F    | CAGCCTTCTTCCTCTGGGTAT        | 105 bp |
| β-actin-R    | CTGTGTTGGCATAGAGGTCTT        |        |
| FASN-F       | AGTGCTTCCCTTTTCACGACT        | 115 bp |
| FASN-R       | GCCTGGGGCTCAATAATAGTAGC      |        |
| SREBP-1-F    | TTTCATTTCTCCGCTCCAA          | 120 bp |
| SREBP-1-R    | TTCCTCAGCAGAAGCAAGAAG        |        |

F and R indicated the forward and reverse primer sequence.

**Table 4. Serum levels of biochemical index for each group (mmol/L)**

| Group   | TC     | HDL    | LDL    | TG     |
|---------|--------|--------|--------|--------|
| NB      | -      | -      | -      | -      |
| Control | 2.55±0.70B | 0.81±0.28B | 1.26±0.83B | 0.83±2.02B |
| NAFLD   | 17.30±2.51A | 7.47±0.34A | 8.30±1.59A | 1.59±0.36B |
| Aged    | 3.23±1.09B | 1.14±0.28B | 1.90±0.55B | 8.37±3.48A |

A/B indicated significant difference (p<0.01)
Fig. 1 Pathology of liver in each group (HE staining ×20).
Fig. 2 Detection result of CpG island in the promoter region of FASN (Part) and SREBP(part). The CpG sites were measured using pyrosequencing, and the “methylation%” was the average of “%” of each CpG site measured. The figure presented the results of methylation rate of sample A4,A5,E1 E2.
Fig. 3 Methylation rate in the promoter regions of FASN and SREBP.

Fig. 3A presented the high methylation rate region of FASN and SREBP gene of each treatment. Fig. 3B showed the low methylation rate region of the FASN gene and SREBP gene of each treatment. The letters "A / B/C" above each column indicated significant difference (p<0.01).
Fig. 4 Transcription levels of SREBP and FASN (qPCR). The transcriptional level of FASN gene for newborn, aged, NAFLD, and normal gerbils was graphed in Fig. 4A. The transcriptional level of SREBP gene for newborn, aged, NAFLD, and normal gerbils was presented in Fig. 4B.
Fig. 5 Expression levels of SREBP and FASN (Western blot analysis). Fig. 5A showed the western blot results of FASN and SREBP genes for each treatment. Fig. 5B outlined the average grayvalue of each treatment compared with β-actin.