Author's response to reviews

Title: High-saturated fat diet delays diethylnitrosamine-induced hepatocellular carcinoma

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Author's response to reviews: see over
A point-by-point reply to reviewers’ comments

**Manuscript title:** High-Saturate-Fat Diet Delays Development of Diethylnitrosamine-induced Hepatocellular Carcinoma

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**Comments and suggestions from Reviewer 1 (Steven Dooley)**

**Comments/Suggestions (Reviewer 1.1):** Authors showed that high fat diet can delay HCC development in a rat DEN model. The finding is very interesting and important for HCC research. The current finding is contrary to the finding from Hill-Baskin AE et al, Hum Mol Genet. 2009. Park EJ et al Cell. 2010, Blanche C. Ip et al. The Journal of Nutrition. 2014. However, different models were used. In this paper, authors used a rat DEN HCC model as contrasting with mice DEN HCC models in the other above-mentioned papers. Further, a different genetic background might cause different responses to high fat diet. But, still several major concerns are relevant. These papers should be carefully discussed in the context of the authors’ results.

In the current paper, the authors showed that high fat diet can delay HCC. However, the low fat diet group should also be included, to see whether low fat diet could promote HCC. This will be a valuable information to understand how fat diet can affect HCC development in rats.

**Reply:** We have made the revision as reviewer suggested (Page 14). Discussion/comments on discrepancy between our data and the data published in the above-quoted papers have been added to the relevant portion of this revised manuscript. In our study, normal control diet (NCD) mice were fed standard chow consisting of 13.8% calories from fat, 60.5% calories from carbohydrates, and 25.7% calories from protein, and the high-fat diet (HFD) mice received 10% lard oil, 2% cholesterol and 88% standard chow, with 36.5% calories from fat, 44.6% calories from carbohydrates, and 18.9% calories from protein for 10, 12, and 14 weeks, respectively. As this study mainly focuses on the impact of HFD on the development of HCC, the low-fat diet (<5% of the energy from fat) arm was not included. As a result, all comparisons were made between the NCD and HFD groups. Indeed, a recently published study have revealed that a low-fat intake could lead
to the deterioration of energy status in HCC patients, and this was associated with a poor recovery from invasive treatments (Yamada K et al. Nutr J 2013; 12: 79).

**Comments/Suggestions (Reviewer 1.2):** Authors showed reduced proliferation and increased apoptosis in high-fat diet DEN mice at 10 and 12 weeks. Here, more molecular details should be addressed to find out the mechanism behind the observation. In figure 3, at week 14, apoptosis rate in high fat diet DEN mice is reduced instead of decreased. This should be clarified.

**Reply:** These comments are very good ideas to improve the quality of the manuscript. In fact, we have detected various molecular details behind these observations. As compared to that of DEN+NCD group, rats in the DEN+HFD group demonstrated higher activity of serum SOD before 12 weeks. In contrast, the DEN+HFD group exhibited hepatic MDA content much lower than that of DEN+NCD group. Additionally, the mRNA levels of IRS-1 and IGF-I were significant higher in the liver of DEN+NCD group rather than DEN+NCD group after the HFD exposure for 10, 12, and 14 weeks. Serum levels of adiponectin and CK-18, and hepatic expression of LC3 and Beclin-1 were also affected by the treatment of HFD. However, present studies are not sufficient to uncover the mechanisms underlying HFD treatment. It is true that apoptosis rate in HFD+DEN mice is significantly increased at the time points of 10, 12 week, whereas reduced at the time point of 14 week. As shown in figure 4, we have display the dynamic changes of the apoptosis in the revised manuscript.

**Comments/Suggestions (Reviewer 1.3):** In figure 1, pictures of the whole liver should be provided to show the difference.

**Reply:** The pictures of the whole livers from NCD and HFD with DEN groups at the end of 10,12, and 14 weeks were shown in supplement figure at the end of the reply, and the representative photos from each group have been added to the revised Figure 2.

**Comments/Suggestions (Reviewer 1.4):** In figure 2a, data from all individual mice should be shown as immunoblot for PCNA.

**Reply:** We have made the revision as reviewer suggested. Data from all mice have been shown as immunoblot for PCNA (Figure 3A), and immunohistochemistry for PCNA (Figure 3B).

**Comments/Suggestions (Reviewer 1.5):** In figure 2a and 3, immunohistochemistry staining of PCNA and Caspase 3 should be shown in order to find out the cell type and expression area in the
animals. Proliferation markers such as ki67 or Tunel staining should also be provided.

**Reply:** We have tested the hepatic expression of PCNA, TNF-α, and IL-6 at the end of 10, 12, 14 weeks as the reviewer suggested. For the sake of time limitation for revision, further exploration of the mechanisms underlying DEN and HFD treatment have not done in the present study.

**Comments/Suggestions (Reviewer 1.6):** In tables 2 and 3, representative photos of fibrosis and differentiation should be included. Furthermore, inflammation should also be analyzed. Park EJ et al Cell. 2010 showed that enhanced production of IL-6 and TNF is import for high fat diet induced HCC in mice. Here, the authors should find out whether the situation is different in rats and include mRNA/ELISA data for IL6 and TNFa.

**Reply:** We have made the revision as reviewer suggested. Representative photos of liver fibrosis as assessed by VG stain and differentiation degree of heptocellular carcinoma have been added to the Figure 1. mRNA levels of TNF-α and IL-6 have been analyzed in the liver tissue. Chronic hepatitis activity index (HAI) proposed by Knodell was also adopted to calculate the liver inflammatory activity score in the revised manuscript. Therefore, we could analyze the impact of inflammation on the initiation of HCC in DEN mice fed with or without HFD. Our findings are different from the study by Park EJ et al, as the expression of inflammatory cytokines and pathological scoring in the DEN+HFD group is significantly less than that in the DEN+NCD group at the end of 10, 12 weeks, even at the end of 14 weeks. More details see the below table.

<table>
<thead>
<tr>
<th>Time</th>
<th>Group</th>
<th>N</th>
<th>HAI</th>
</tr>
</thead>
<tbody>
<tr>
<td>10W</td>
<td>DEN+NCD</td>
<td>7</td>
<td>12.1±2.2</td>
</tr>
<tr>
<td></td>
<td>DEN+HFD</td>
<td>7</td>
<td>10.7±2.9</td>
</tr>
<tr>
<td>12W</td>
<td>DEN+NCD</td>
<td>7</td>
<td>14.9±4.5</td>
</tr>
<tr>
<td></td>
<td>DEN+HFD</td>
<td>7</td>
<td>11.0±1.2(^\triangle)</td>
</tr>
<tr>
<td>14W</td>
<td>DEN+NCD</td>
<td>9</td>
<td>15.1±3.1</td>
</tr>
<tr>
<td></td>
<td>DEN+HFD</td>
<td>10</td>
<td>14.0±2.3</td>
</tr>
</tbody>
</table>

\(^\triangle\) \(P<0.05\)

**Comments/Suggestions (Reviewer 1.7):** How many animals were used per time point in each of the analyses? This should always be stated in the figures.

**Reply:** This point has been clarified in the revised manuscript.

**Minor Essential Revisions**

**Comments/Suggestions (Reviewer 1.8):** …..missing labels on figures, the wrong use of a term,
spelling mistakes. In figure 2a, molecular weight of PCNA and actin should be mentioned on the picture.

Reply: Necessary corrections/revisions have been done.

Comments/Suggestions (Reviewer 1.9): In figure 2b, 2c and 3, dot graph would be better than column to show individual animals.

Reply: We have made the revision as reviewer suggested. Because of the similar information obtained from ELISA and western blot, the ELISA of PCNA has been replaced by immunohistochemical staining.

Comments/Suggestions (Reviewer 1.10): Reference 19 and 33 is exactly the same.

Reply: Redundant reference has been removed.
Comments and suggestions from Reviewer 2 (Hirofumi Uto)

Comments/Suggestions (Reviewer 2.1): The effect of HFD in this study may depend on total calorie intake rather than fat intake. In fact, HFD restored malnutrition in the DEN-treated rats as stated by authors. Authors should justify the total calorie intake in the HFD+DEN group and NCD+DEN group.

Reply: We can not exclude that the observed effect of the HFD was exclusively due to high fat intake, as the rats in the DEN+HFD group had more daily food intake than those in the DEN + NCD group (Table 1). However, as each diet contains the same amount of total calorie, we believe the observed effect could be mostly attributed to the difference in the dietary fat between these 2 diets, and partly related to the total calorie intake.

Comments/Suggestions (Reviewer 2.2): Rahman et al concluded that the effect of dietary fat during the initiation phase of AOM-induced hepatocarcinogenesis depends on the type of fat and its fatty acid composition (Ref 18). Sugie et al also showed that the density and the unit area of AOM-induced enzyme altered foci in the liver were significantly lower in the high fish oil group than in the 5% corn oil group and the low fish oil group (Nutr Cancer. 1995;24:187-95.). In addition, Rahman et al reported that fish oil rich in polyunsaturated omega-3 fatty acids could inhibit DEN-induced hepatocarcinogenesis in rats (Jpn J Cancer Res. 1999;90:31-9.). If the effect of HFD in this study depends on fat rather than total calorie intake, authors should clarify whether high-saturate-fat or high-unsaturated-fat is better to inhibit DEN-induced hepatocarcinogenesis.

Reply: In the original experimental design, we aimed to study the effect of saturated fatty acids on DEN-induced hepatocarcinogenesis. The authors appreciate the suggestion of this reviewer that the effect of unsaturated fatty acids should also be investigated. This theme would be our future research topic.

Comments/Suggestions (Reviewer 2.3): Authors showed the anti-proliferative and pro-apoptotic effect of HFD in rat liver. Are these effects observed in both tumor cells and non-tumor cells (normal hepatocytes)?

Reply: Yes, the presented data were derived from tumor cells and non-tumor cells.

Comments/Suggestions (Reviewer 2.4): There was an increase in the hepatic level of caspase-3 in the HFD+DEN group compared to the NCD+DEN group at weeks 10 and 12, and authors
concluded that anti-apoptotic effect may be associated with the attenuation of hepatocarcinogenesis. However, the hepatic level of caspase-3 in the HFD+DEN group was significantly lower than that of the NCD+DEN group at weeks 14.

**Reply:** Please refer to the Reply to Comments/Suggestions (Reviewer 1.5).

**Comments/Suggestions (Reviewer 2.5):** Authors stated that there was a more significant reduction in the PCNA expression in HFD+DEN group at weeks 12 and 14 compared to rats in the NCD+DEN group (page 7, line 23-). However, the PCNA expression (hepatic content of PCNA) in HFD+DEN group at weeks 14 was relatively higher than those in the NCD+DEN group (Fig 2 C).

**Reply:** Necessary rewording has been done in the revised manuscript. Please also refer to the Reply to Comments/Suggestions (Reviewer 1.5).

**Comments/Suggestions (Reviewer 2.6):** Authors stated that HFD appeared to attenuate the occurrence of HCC and malignant differentiation in rat HCC model induced by DEN (Page 9, 12-). Three steps of progression of hepatocarcinogenesis have been proposed: initiation, promotion, and progression. Authors should discuss this point more in detail.

**Reply:** Appropriate discussion to this stepwise hepatocarcinogenesis model has been added to the relevant portion of the revised manuscript.

**Comments and suggestions from Reviewer 3 (Alexander Wree)**

**Comments/Suggestions (Reviewer 3.1):** Table 1: Values for TC, TG, HDL-C, LDL-C are not in line with the rest of the table.

**Reply:** We have made the revision as reviewer suggested.

**Comments/Suggestions (Reviewer 3.1):** The abstract provided does not give any background and introduction to the topic. The sentence given with the heading background describes the aim of the study. Moreover, authors introduce the abbreviation DNE for diethylnitrosamine.

**Reply:** The abstract has been revised and the wrongly spelled words have been corrected.

**Comments/Suggestions (Reviewer 3.2):** Based on which rationale did the authors choose the time points 10, 12, and 14 weeks? DEN has been used for a broad range of time points in rats anywhere from 8 weeks to 16 months (e.g. Taya et al 2014, Carthew et al 1997, Takahashi et al. 1984).

**Reply:** The impact of HFD on DEN-induced hepatocarcinogenesis was dynamically studied.
We chose week 10 as the starting point and week 14 as the endpoint of analysis. This is because all rats developed HCC by week 14, and the effect of HFD on liver pathology becomes evident during this time period. In addition, we have done the experiment of 16 weeks of DEN with and without HFD before choosing the time points, maintaining the experimental rats beyond week 16 was not ethically permitted because the general condition of all rats deteriorates thereafter.

**Comments/Suggestions (Reviewer 3.3):** Authors extensively describe the liver histology in mice fed with NCD and HFD with or without DEN treatment. Therefore, macroscopic images of livers should be presented in Figure 1 along with microphotographs in high, as well as in low magnifications.

**Reply:** Please refer to the Reply to Comments/Suggestions (Reviewer 1.3).

**Comments/Suggestions (Reviewer 3.4):** Did the authors perform any specific staining to assess liver fibrosis, e.g. Sirius Red Staining or Masson's trichrome? If so, this should be presented to the readership.

**Reply:** Data from VG staining for liver fibrosis were added. Please refer to Reply to Comments/Suggestions (Reviewer 1.6).

**Comments/Suggestions (Reviewer 3.5):** Authors state in the Method section that a commercial enzyme-linked immunosorbent assay (ELISA) kit was used to quantify the active form of Caspase 3. However, in the Results, as well as the Figures, they report the total content of Caspase 3. Authors emphasize the resistance to apoptotic cell death as an important contributor to the documented phenotype. How do authors explain that the hepatic content of Caspase 3 is increased in measurements at week 10 and 12, while decreased at 14 weeks in the HFD+DEN group when compared to the NCD+DEN group?

**Reply:** This aspect has been discussed in the Discussion of the revised manuscript. Please also refer to the Reply to Comments/Suggestions (Reviewer 1.5).

**Comments/Suggestions (Reviewer 3.6):** In previous studies, DEN has been administered via gavage, peritoneal injection, or tail vein injection. The main effect reported in the presented study is addressed to a dietary intervention. How can the authors exclude that the diet itself does not interfere with the DEN? Is the beneficial effect of HFD still present when DEN is administered via a non-oral path?
Reply: In this study, DEN was administered by gavage and the impact of HFD on DEN-induced hepatocarcinogenesis was examined. The impact of other dietary components on the carcinogenic effect of DEN as well as the effect of different routes of administration on the combinatorial effect of HFD and DEN are beyond the scope of this study. These ideas may form good rationales for future studies.

Comments/Suggestions (Reviewer 3.7): Page 5, line 1: “Metavir Score system”. An appropriate reference should be added.


Comments/Suggestions (Reviewer 3.8): Page 6, line 10: “as shown in Table 4”, do the authors mean Table 1 or do they refer to additional table.

Reply: This confusion has been clarified in the revised manuscript.

Comments/Suggestions (Reviewer 3.9): Figures 2 and 3: Number of mice per group analysed in panel B and C should be given.

Reply: The number of mice per group analysed in Figures 2 and 3 have been given in the revised manuscript.
1. Liver gross observation in DEN+CD group at the end of 10 weeks

2. Liver gross observation in DEN+HFD group at the end of 10 weeks
3 Liver gross observation in DEN+CD group at the end of 12 weeks

4 Liver gross observation in DEN+HFD group at the 12 weeks

5 Gross liver pictures of the NCD+DEN group at week 14
5 Gross liver pictures of the HFD+DEN group at week 14

6 Normal liver at the 14 weeks
6 Fatty liver at the 14 weeks