Immunohistochemical expression of autophagosome markers LC3 and p62 in preneoplastic liver foci in high fat diet-fed rats

Sosuke Masuda¹, Sayaka Mizukami¹,², Ayumi Eguchi¹, Ryo Ichikawa¹, Misato Nakamura¹, Kazuki Nakamura¹, Rena Okada¹, Takaharu Tanaka¹, Makoto Shibutani¹ and Toshinori Yoshida¹

¹Laboratory of Veterinary Pathology, Tokyo University of Agriculture and Technology, 3-5-8 Saiwai-cho, Fuchu-shi, Tokyo 183-8509, Japan
²Pathogenetic Veterinary Science, United Graduate School of Veterinary Sciences, Gifu University, 1-1 Yanagido, Gifu-shi, Gifu 501-1193, Japan

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ABSTRACT — Nonalcoholic fatty liver disease (NAFLD) is characterized by excessive deposition of droplets in hepatocytes. Patients with NAFLD can be at risk for nonalcoholic steatohepatitis, which can lead to hepatocellular carcinoma. Autophagy is a cellular pathway that is crucial for survival and homeostasis, and which protects against pathophysiological changes like obesity and cancer. We determined the expression of autophagy markers in preneoplastic hepatic lesions and the effects of an autophagy repressor chloroquine (CQ) or inducer amiodarone (AM) in a steatosis-related hepatocarcinogenesis model. Male F344 rats were fed a control diet or high fat diet (HFD), and subjected to initiation and promotion steps with N-nitrosodiethylamine injection at week 0 and a partial hepatectomy at week 3. Several HFD-fed rats were administered 0.1% CQ and 0.5% AM in their drinking water during week 2 and 8. CQ and AM did not improve HFD-induced obesity. AM, but not CQ, significantly decreased the number of glutathione S-transferase placental form-positive preneoplastic liver foci in the liver. Autophagosome markers LC3 and the LC3-binding protein p62 were heterogeneously expressed in the preneoplastic foci. CQ might inhibit autophagy by significantly increased p62/LC3 ratio, while AM might have a potential of inducing autophagy by showing an increased gene expression of the autophagy regulator, Atg5. These results suggest that preneoplastic lesions express autophagosome markers and that AM might decrease steatosis-related early hepatocarcinogenesis by potentially inducing autophagy in HFD-fed rats, while inhibition of autophagy by CQ did not alter the hepatocarcinogenesis. However, an immunohistochemical trial revealed a technical limitation in detecting autophagosome markers because there were variations in each preneoplastic lesion.

Key words: Amiodarone, Autophagy, NAFLD, LC3, p62, Preneoplastic lesion

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is characterized by excessive deposition of droplets in hepatocytes. Some patients with NAFLD are at risk for nonalcoholic steatohepatitis (NASH) through inflammation and lipotoxicity, followed by hepatic cirrhosis and hepatocellular carcinoma (HCC) (Doulberis et al., 2017). For patients with NAFLD or NASH, the risk of HCC increases with age (Sheedfar et al., 2013) and their incidences, including liver deaths, are estimated to gradually increase from 2015 to 2030 (Estes et al., 2018). In experimental rodents, a high fat diet (HFD) is reproducibly able to induce steatosis similar to NAFLD or NASH (Van Herck et al., 2017), and NASH-associated HCC models using the liver initiator N-nitrosodiethylamine (DEN) have been developed (Wu, 2016). Recently, our group (Yoshida et al., 2017) developed a steatosis-related early hepatocarcinogenesis model in HFD-fed rats based on a medium-term liver assay for 8 weeks (Ito et al., 2003). The HFD-induced
hyperlipidemia and steatosis and preneoplastic lesions in the liver, and the effects on the liver were related to altered lipid metabolism and oxidative stress (Murayama et al., 2018; Nakamura et al., 2018).

These experimental models can be used to evaluate preneoplastic lesions with surrounding steatosis during relatively shorter periods to identify compounds with therapeutic and preventive prowess. Therapeutic approaches on insulin resistance, hyperlipidemia, lipotoxicity, inflammation, and oxidative stress have shown promising effects for NAFLD (Ibrahim et al., 2013). Autophagy is involved in attenuating inflammation and liver injury. Thus, autophagy may be a potential therapeutic approach in NAFLD (Mao et al., 2016). Autophagy is the major intracellular degradation system by which cytoplasmic materials are enclosed by autophagosomes and delivered to the lysosome for degradation. Autophagy serves as a critical recycling system that produces energy for cellular renovation and homeostasis (Mizushima and Komatsu, 2011). Multiple Atg proteins, including Atg5 and Atg7, regulate autophagosome formation, and p62 is one of the best characterized substrates of selective autophagy. p62 directly interacts with LC3 on the isolated membrane through the LC3-interacting region, and subsequently p62 is incorporated into the autophagosome and then degraded (Mizushima and Komatsu, 2011). The cellular process is known as autophagy flux. It includes autophagosome formation, maturation, fusion with lysosomes, and subsequent breakdown to release macromolecules into the cytosol (Zhang et al., 2013). The process is sensitive to induction by starvation and other stresses, and the impairment or activation of autophagy contributes to pathogenesis of diverse diseases, including hepatic steatosis and HCC (Mizushima and Komatsu, 2011). Autophagy regulates lipid metabolism and the inhibition of autophagy increases steatosis in vitro and in vivo (Singh et al., 2009). Accumulating experimental and clinical data suggest that autophagy is involved in the pathogenesis of NAFLD and NASH (Lavallard and Gual, 2014). In HFD-fed mice and NASH patients, expression of p62 and LC3 was increased in liver samples (González-Rodríguez et al., 2014; Zhang et al., 2016). Atg7 knockdown can reverse the ameliorated liver steatosis in db/db mice (Xiao et al., 2016) and Atg7 or -5 knockdown reportedly increases benign liver tumors in mice (Takamura et al., 2011; Tian et al., 2015).

In this study, to elucidate the roles of autophagy in NAFLD/NASH-related hepatocarcinogenesis, we determined the expression of the autophagosome markers LC3 and p62 in preneoplastic liver foci, together with gene expression analysis for several autophagy-related genes, including Atg5 and Atg7, and the effects of an autophagy inducer or repressor were assessed in a steatosis-related hepatocarcinogenesis model (Yoshida et al., 2017; Murayama et al., 2018; Nakamura et al., 2018).

MATERIALS AND METHODS

Chemicals

Chlorochine diphosphate (CAS No.50-63-5, purity: > 96%), amiodarone hydrochloride (CAS No. 19774-82-4, purity: > 98%), and DEN (CAS No. 55-18-5, purity > 99%) were purchased from Wako Pure Chemical Ltd. (Osaka, Tokyo), Sigma-Aldrich Co. LLC. (St. Louis, MO, USA), and Tokyo Kasei Kogyo (Tokyo, Japan), respectively.

Animals and treatment

Forty two 5-week-old male F344/N rats purchased from Japan SLC, Inc. (Shizuoka, Japan) were maintained in a room with a controlled environment (room temperature, 23 ± 3°C, relative humidity, 50 ± 20%) with alternating 12-hr light/dark cycles. The rats had free access to a control diet (D12450K; Research Diets, Lane, NJ, USA) and tap water. After a 1-week acclimatization period, a medium-term liver carcinogenesis bioassay (Ito et al., 2003) was conducted as follows. All animals received an intraperitoneal injection of DEN at a dose of 200 mg/kg body weight and were divided into four groups. The control (CTL) group was fed a control diet (D12450K, n = 10). The other groups were fed a HFD (D12451; Research Diets) alone (HFD group, n = 11) or the diet with 0.1% CQ (HFD+CQ group, n=11) or 0.5% AM (HFD+AM group, n = 10) in drinking water for 6 weeks beginning 2 weeks after DEN initiation to the end of the study. All animals were subjected to a two-thirds partial hepatectomy at week 3. The dose selection of CQ and AM was based on previous studies (Almeida et al., 2008; Lezmi et al., 2013), and a preliminary study using 0.05% or 0.1% CQ and 0.25% or 0.5% AM in drinking water. During the study, clinical signs were observed daily, and body weight as well as food and water intake were measured once a week. At week 8, the rats were euthanized by exsanguination in a carbon monoxide atmosphere after overnight fasting to reduce glycogen and induce autophagy. The liver as well as abdominal fat tissue surrounding the spermatic cord were excised at necropsy and weighed. Sections of liver tissue were fixed using 4% paraformaldehyde in 0.1 M phosphate-buffered saline (pH 7.4) for histopathology and immunohistochemistry. Liver pieces were put in sample tubes, frozen in dry ice, and stored at −80°C until needed for further analysis. All procedures were conducted in compliance with the Guidelines for
Proper Conduct of Animal Experiments (Science Council of Japan, June 1, 2006) and were done according to the protocol approved by the Animal Care and Use Committee of the Tokyo University of Agriculture and Technology.

Histopathology and immunohistochemistry
Liver sections were routinely dehydrated in graded ethanol, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E) for histopathological examination. The pathological changes, including the presence of steatotic cells, ballooning cells, and lobular inflammation, were graded by the NAFLD activity score (NAS) (Kleiner et al., 2005; Itagaki et al., 2013; Nakamura et al., 2018). Immunohistochemical staining of adipophilin (ADFP), glutathione S-transferase placental form (GST-P), Ki-67, MAP1LC3A (LC3), and p62/SQSTM1 (p62) was performed under the conditions shown in Supplemental Table 1. The deparaffinized liver sections were treated as previously reported (Yoshida et al., 2017). The numbers and areas of GST-P-positive foci (> 0.2 mm-diameter) and the total areas of the liver sections were quantified using Scion Image (Scion Corp., Frederick, MD, USA) as previously described (Ito et al., 2003). Ki-67-positive cells were examined in over 1000 cells within GST-P-positive foci. The expression of LC3 or p62 in GST-P-positive foci per total liver areas was enumerated in serial sections. The ratio of the number of p62-positive foci to the number of LC3-positive foci in GST-P-positive foci was shown as p62/LC3 ratio. The numbers of Ki-67-, LC3-, or p62-positive hepatocytes or Kupffer cells in the surrounding parenchyma (outside GST-P-positive foci) were measured in five or 10 high power fields at 400 × magnification.

Real-time reverse transcription-polymerase chain reaction analysis
Analysis of mRNA levels of the genes listed in Supplemental Table 2 in liver tissues (n = 6 animals per group) was performed with real-time reverse transcription-polymerase chain reaction (RT-PCR) as previously reported (Murayama et al., 2018). The relative differences in gene expression were calculated using the threshold cycle (CT) values that were first normalized to those of the hypoxanthine phosphoribosyl transferase 1 (Hprt1) gene, the endogenous control in the same sample, and then relative to a control CT value using the 2−ΔΔCT method (Livak and Schmittgen, 2001).

Statistical analyses
All data are expressed as mean ± standard deviations. The statistical significance of the differences between the control and the treated group(s) was determined the Tukkey or Steel-Dwass multiple comparison test. A p-value < 0.05 was considered statistically significant.

RESULTS
Chloroquine and amiodarone do not affect body weight, food and water intake, and organ weight
Treatment with CQ or AM did not affect body weight change in HFD-fed rats (Supplemental Table 3). Food and water intake were comparable with the control group in the HFD group with or without CQ or AM. The HFD significantly increased absolute and relative adipose tissue weights, independent of CQ or AM treatment. The HFD with or without CQ or AM treatment did not affect absolute and relative liver weights as compared to the CTL group.

HFD increases NAFLD score activity, including ballooning change, in the liver
Overnight fasting decreased glycogen deposition in the hepatocytes in all the groups, as shown by H&E staining, when compared with the liver in our previous study using non-fasting rats (Yoshida et al., 2017). However, steatosis persisted in each group and immunostaining for adipophilin was positive. By scoring steatosis, ballooning change and lobular inflammation, the HDF increased the total score NAS in the HFD, HFD+CQ, and HFD+AM groups, and the effects were significantly different from the CTL group in the HFD and HFD+AM groups (Table 1). Ballooning change, but not steatosis and lobular inflammation, significantly increased in the HFD group with or without CQ or AM treatment when compared to the CTL group. The HFD+CQ group tended to decrease the scores of steatosis and lobular inflammation when compared with the HFD group, although there were no significant differences.

Early hepatocarcinogenesis parameters
The HDF increased the number, but not area, of GST-P-positive liver foci. The change was not statistically significantly different from the CTL group (Fig. 1, 2A, 2B). Treatment with AM, but not CQ, decreased the number and area of GSTP-positive foci when compared with the CTL group and HFD group. This effect on the number was significantly different.

In the hepatocytes inside the liver foci, the cell proliferation marker Ki-67 was significantly increased in the HFD group when compared with the CTL group, and tended to decrease in the HFD+AM group when com-
### Table 1. NAFLD activity score in the liver.

<table>
<thead>
<tr>
<th>Group</th>
<th>CTL</th>
<th>HFD†</th>
<th>HFD+CQ‡</th>
<th>HFD+AM$</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of animals</td>
<td>10</td>
<td>10</td>
<td>11</td>
<td>10</td>
</tr>
<tr>
<td>NAFLD activity score (NAS)</td>
<td>2.9 ± 1.2</td>
<td>4.5 ± 1.4a</td>
<td>4.4 ± 1.2</td>
<td>5.0 ± 1.4a</td>
</tr>
<tr>
<td>Steatosis</td>
<td>1.6 ± 1.0</td>
<td>1.8 ± 0.8</td>
<td>1.6 ± 0.8</td>
<td>2.1 ± 0.7</td>
</tr>
<tr>
<td>Hepatocyte ballooning</td>
<td>0.3 ± 0.5</td>
<td>1.6 ± 0.7a</td>
<td>1.7 ± 0.6a</td>
<td>1.5 ± 0.7a</td>
</tr>
<tr>
<td>Lobular inflammation</td>
<td>1.0 ± 0.0</td>
<td>1.2 ± 0.6</td>
<td>1.0 ± 0.0</td>
<td>1.4 ± 0.7</td>
</tr>
</tbody>
</table>

Abbreviations: AM, amiodarone; CTL, control (basal diet); CQ, chloroquine; HFD, high fat diet; NAFLD; nonalcoholic fatty liver disease. Data are shown as the mean ± standard deviation.

†: One rat was eliminated because of severe bile duct proliferation in the liver.
‡: Rats were given HFD with CQ in drinking water during the study.
$: Rats were given HFD with AM in drinking water during the study.
a: p < 0.05 vs CTL (Tukey’s or the Steel-Dwass test).

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**Fig. 1.** Representative images of immunohistochemistry for GST-P, LC3 and p62 in the liver of a rat fed the HFD with or without AM in drinking water. GST-P-positive foci contained LC3- and p62-positive cells in a rat fed the HFD. In a rats fed with HFD with AM in drinking water, GST-P-positive foci contained LC3-positive cells, but p62-positive cells were not found. Bar = 100 μm.
pared with the HFD group (Fig. 2C). A similar effect of AM on Ki-67 expression outside the foci was detected (Table 2). The autophagosome marker LC3 expressed hepatocytes inside and outside the liver foci, and expression was prominent in the liver foci (Fig. 1). The expression rate of LC3 appeared to decrease in the HFD+CQ group when compared with the HFD group, and the change was not significantly different (Fig. 2D). In hepatocytes and Kupffer cells outside the liver foci, expression of LC3 appeared to increase in the HFD and HFD+AM groups, and decrease in the HFD+CQ group; in Kupffer cells, a statistically significant difference was detected in the HFD+CQ group compared with the CTL and HFD groups (Table 2). The LC3-binding protein p62 was also expressed in the hepatocytes inside the liver foci (Fig. 1); the expression rate appeared to increase in the HFD group with no significant changes, but an increasing trend was not observed in the HFD+CQ and HFD+AM groups compared with the HFD group (Fig. 2E). The p62/LC3 ratio appeared to increase in the HFD group when compared with the CTL group, and significantly increased in the HFD+CQ group (Fig. 2F). The ratio in the HFD+CQ group appeared to increase when compared with the HFD group, but the change was not significantly different. The ratio in the HFD+AM group was significantly decreased when compared with the HFD+CQ group and similar to that in the CTL group. In the hepatocytes outside the liver foci, treatment with CQ or AM decreased p62-positive hepatocytes when compared with the CTL and HFD groups, but these changes were not statistically significantly different (Table 2).

Gene expression analysis

With autophagy-related genes, expression of Atg5, but not Atg7, Lc3, Lamp1, and Lamp2, was significantly increased in the HFD+AM group when compared with the CTL group (Table 3). With lipid metabolism-related genes, the expression of the fatty acid synthetase gene, Fasn, was significantly decreased in the HFD group with or without CQ or AM treatment (Supplemental Table 4). The expression of the cholesterol transporter gene, Abca1, was significantly increased in the HFD+CQ group when compared with the CTL and HFD groups. The expression of the fatty acid desaturation gene, Scd, was significantly decreased in the HFD+CQ and HFD+AM groups when compared with the CTL group. With lipid droplet-related genes, there were no statistically significant differences between each group. With oxidative stress-related genes, expression of Gpx2 was increased in the HFD, HFD+CQ, and HFD+AM groups, when compared with the CTL group; however, these changes were not statistically significantly different.

Fig. 2. Quantitative analysis of GST-P-positive foci with expression of Ki-67, LC3, and p62, and the p62/LC3 ratio. Quantitative analysis of the number (A) and area (B) of GST-P-positive foci; (C) Ki-67 labeling index; (D, E) LC3 and p62 expression rate; (F) p62/LC3 ratio. Columns represent mean ± standard deviation.* † ‡ p < 0.05 versus CTL, HFD and HFD+CQ, respectively (Tukey or Steel-Dwass multiple comparison test).
DISCUSSION

The present study demonstrates the expression of the autophagosome markers LC3 and the LC3-binding protein p62 in liver tissues with steatosis and preneoplastic lesions in HFD-fed rats when treated with an autophagy repressor (CQ) or inducer (AM). Both drugs are widely used to understand the roles of autophagy. CQ is an anti-malaria drug and immunosuppressant, which represses autophagy by inhibiting binding of autophagosomes to lysosomes (Cooper and Magwere, 2008; Jover et al., 2012). AM is a nonselective ion channel blocker and well-established anti-arrhythmic drug (Julian et al., 1997; Morse et al., 1988), which induces autophagy by mitochondrial toxicity though Ca^{2+} influx (Lin et al., 2015). The present study demonstrated that 1) AM was more effective than CQ under the present study protocol for hepatocarcinogenesis, 2) analyses with LC3 and p62 might be useful to investigate autophagy in the preneoplastic lesions compared with the surrounding hepatocytes, and 3) the p62/LC3 ratio could be valuable in assessing autophagy in the preneoplastic lesions. However, an immunohistochemical trial revealed a technical limitation in detecting autophagosome markers because there were variations in each preneoplastic lesion, resulting in no significant difference between each group.

To elucidate the effects of the HFD feeding, we initially examined the histopathology of liver tissue using the NAS scoring system (Kleiner et al., 2005) with gene expression analysis for lipid metabolism and oxidative stress. NAS is suitable to evaluate NAFLD and NASH in the liver obtained from patients and rodent models (Itagaki et al., 2013; Nakamura et al., 2018). The HFD increased NAS with ballooning changes in the liver sections, although there was no significant change in steatosis. The ballooning change of hepatocytes is the most important histological indicator to confirm hepatocyte degeneration by the excessive deposition of fat droplets (Kleiner et al., 2005). The lack of increase in the steatosis score might be due to a time-course alteration of lipid deposition in hepatocytes in HDF-fed rats. The liver becomes rapidly (1-2 weeks) infiltrated with lipids with the lipid content then decreasing in subsequent weeks (weeks 4-6), with subsequent slow re-infiltration for a longer period of time (6-16 weeks) (Gauthier et al., 2006). The HFD reduced the mRNA level of the fatty acid synthetase gene.

Table 2. Expression of Ki–67, LC3 and p62 in the surrounding hepatocytes other than preneoplastic liver foci.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Distribution</th>
<th>CTL (No/5HPF)</th>
<th>HFD (No/5HPF)</th>
<th>HFD+CQ (No/5HPF)</th>
<th>HFD+AMS (No/5HPF)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ki-67</td>
<td>outside (hepatocyte)</td>
<td>23.1 ± 6.1</td>
<td>23 ± 9.7</td>
<td>27.8 ± 6.9</td>
<td>15.2 ± 6.3</td>
</tr>
<tr>
<td>LC3</td>
<td>outside (hepatocyte)</td>
<td>1.8 ± 1.1</td>
<td>3.4 ± 1.8</td>
<td>1.5 ± 1.1</td>
<td>5.0 ± 3.9</td>
</tr>
<tr>
<td></td>
<td>outside (Kupffer cell)</td>
<td>58.0 ± 24.6</td>
<td>63.1 ± 16.6</td>
<td>17.2 ± 14.9</td>
<td>66.5 ± 45.9</td>
</tr>
<tr>
<td>p62</td>
<td>outside (hepatocyte)</td>
<td>2.2 ± 1.8</td>
<td>2.3 ± 2.8</td>
<td>1.1 ± 1.1</td>
<td>0.6 ± 0.9</td>
</tr>
<tr>
<td>p62/LC3</td>
<td>outside (hepatocyte) (Ratio)</td>
<td>0.66 ± 0.56</td>
<td>0.37 ± 0.46</td>
<td>0.38 ± 0.52</td>
<td>0.15 ± 0.31</td>
</tr>
</tbody>
</table>

Abbreviations: AM, amiodarone; CTL, control (basal diet); CQ, chloroquine; HFD, high fat diet; HPF, high power field. Data are shown as the mean ± standard deviation.
‡: Rats were given HFD with CQ in drinking water during the study.
$: Rats were given HFD with AM in drinking water during the study.
a: p < 0.05 vs CTL (Tukey’s or the Steel-Dwass test).
b: p < 0.05 vs HFD (Tukey’s or the Steel-Dwass test).

Table 3. Gene expression analysis of autophagy in liver samples.

<table>
<thead>
<tr>
<th>Autophagy-related genes</th>
<th>CTL</th>
<th>HFD</th>
<th>HFD+CQ</th>
<th>HFD+AMS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atg5</td>
<td>1.02 ± 0.22</td>
<td>1.27 ± 0.09</td>
<td>1.23 ± 0.21</td>
<td>1.51 ± 0.21</td>
</tr>
<tr>
<td>Atg7</td>
<td>1.02 ± 0.21</td>
<td>1.13 ± 0.21</td>
<td>1.04 ± 0.10</td>
<td>1.21 ± 0.21</td>
</tr>
<tr>
<td>Lamp1</td>
<td>1.01 ± 0.17</td>
<td>0.93 ± 0.18</td>
<td>1.00 ± 0.10</td>
<td>1.09 ± 0.19</td>
</tr>
<tr>
<td>Lamp2</td>
<td>1.02 ± 0.21</td>
<td>0.92 ± 0.16</td>
<td>1.04 ± 0.15</td>
<td>1.16 ± 0.21</td>
</tr>
<tr>
<td>Lc3</td>
<td>1.01 ± 0.20</td>
<td>1.10 ± 0.18</td>
<td>1.10 ± 0.14</td>
<td>1.34 ± 0.27</td>
</tr>
</tbody>
</table>

Abbreviations: AM, amiodarone; CTL, control (basal diet); CQ, chloroquine; HFD, high fat diet. Abbreviations of genes are shown in Supplemental Table 2. Data are shown as the mean ± standard deviation.
‡: Rats were given HFD with CQ in drinking water during the study.
$: Rats were given HFD with AM in drinking water during the study.
a: p < 0.05 vs CTL (Tukey’s or the Steel-Dwass test).
Autophagosome markers in preneoplastic liver foci

Fasn (Softic et al., 2016), when compared with the CTL group. HFD also tended to increase the mRNA level of an antioxidant gene (Gpx2). The results suggested that the HFD might alter lipid metabolism and subsequently increase oxidative stress, resulting in ballooning changes of hepatocytes, even though a significant increase in steatosis was not detected. AM inhibits mitochondria respiratory chains and fatty β-oxidation, thus causing accumulation of reactive oxygen species and fatty acids (Felser et al., 2013). AM tended to increase steatosis, but this was not significantly different as compared with the HFD group. AM reduced the mRNA level of the gene encoding a desaturation enzyme of fatty acids (Scd) (Softic et al., 2016), which could be in response to steatosis of hepatocytes. The effects of CQ on steatosis have not been examined. However, the drug reduces the mRNA level of Scd and increases the mRNA level of the gene encoding the transporter for cholesterol efflux (Abca1) (Arguello et al., 2015). The results suggest that CQ also altered lipid metabolism under the present study condition, as also shown in the scores of steatosis and related inflammation which might be a decreasing tendency by CQ administration.

Effects of the HFD on GST-P-positive preneoplastic liver foci were variable in our studies, probably reflecting variations due to the study condition and diet lot. Both the area and number of foci were significantly increased in the HDF group compared with the CTL group in our previous study (Murayama et al., 2018) or were both weakly increased without a significant difference in another study (Nakamura et al., 2018). The present study revealed only a minor effect on the number of GST-P-positive preneoplastic liver foci. Interestingly, immunohistochemistry examinations revealed that the HFD specifically increased the expression of the autophagy adaptor, p62, in the liver foci compared with the surrounding hepatocytes, although there were no significant differences. Consistent with this, in NASH patients with HCC and NAFLD mice exposed to DEN, expression of p62 was reported to be mainly restricted to tumor lesions (Chava et al., 2017; Umemura et al., 2016). The main function of p62 is to deliver polyubiquitinated proteins and organelles for autophagosomal-lysosomal degradation, and interference with autophagic flux attenuates p62 degradation (Mizushima and Komatsu, 2011; Zhang et al., 2013). Thus, the observed accumulation of p62 in the liver foci suggests that the HFD might interfere with autophagy by inhibiting the degradation of p62. In NAFLD mice, expression of p62 was also increased in the hepatocytes at an early phase after DEN exposure, followed by a decrease at the chronic phase, suggesting that p62 was not linked to the development of steatotic changes (Umemura et al., 2016). Consistent with this, in NASH patients with HCC, adjacent non-tumorous hepatocytes were negative for expression of p62, similar to normal liver (Chava et al., 2017). We observed a low level expression of p62 in hepatocytes, but not in Kupffer cells, in a similar manner between the CTL and HFD groups.

The HFD also appeared to increase the expression of LC3 in the surrounding hepatocytes in the present study. Compared with p62, expression of LC3 has not been fully elucidated in tumor sections of experimental animals and patients. A mouse xenograft model of cells of the Hep HCC cell line revealed LC3 expression in tumor tissues (He et al., 2016) and accumulation of LC3 in HCC cells, which suggested an induction of autophagy (Cheng et al., 2016). LC3 proteins are cleaved to LC3-I, which is converted to LC3-II via Atg. LC3-II is subsequently relocalized to autophagic vesicles (Mizushima and Komatsu, 2011; Zhang et al., 2013). The increased level of LC3 is not always indicative of autophagy and may represent a blockade of autophagy maturation (Zhang et al., 2013). Although we could not separate LC3-I and LC3-II using immunohistochemistry in the present study, increased expression of p62 with an increase in the p62/LC3 ratio might be interpreted as indicating the inhibition of autophagy at a late step (Zhang et al., 2013). Therefore, a weak increase in HFD-induced preneoplastic liver foci might be mediated by the inhibition of autophagy.

Administration of AM significantly decreased the number of GST-P-positive preneoplastic liver foci, and this was accompanied by decrease of p62 expression and Ki-67 labeling index, although there were no significant differences. Defective autophagy by Atg5 or Atg7 knockdown is usually accompanied by extensive accumulation of p62 (Komatsu et al., 2007), which subsequently develops into hepatocellular adenoma, which can be reduced in size by p62 knockout (Takamura et al., 2011). On the contrary, AM did not alter LC3 expression in preneoplastic liver foci. Previous studies demonstrated that AM can increase cell proliferation activity with a higher level of LC-II in Hep 3B HCC cells (Wu, 2016), and enhance liver regeneration in mice after partial hepatectomy by the induction of autophagy and increased autophagy flux (Lin et al., 2015). In the present study, AM-mediated reduction of p62/LC3 ratio in the preneoplastic foci indicated drug-induced autophagy, with the subsequent decrease in the number of foci. Induction of autophagy by AM was supported by findings of increased expression of Atg5 mRNA in liver samples. Atg proteins, including Atg5, are required to form autophagosomes (Mizushima and Komatsu, 2011).
CQ tended to reduce the expression of p62 and increased the p62/LC3 ratio in the preneoplastic liver foci. The data were consistent with inhibition of autophagy at an early step on the prephagosomal structure or phagophores which is demonstrated by a decrease in LC3 and an increase in p62 (Zhang et al., 2013). The effects of this drug on the preneoplastic liver foci as well as autophagy were obscure in the present study. Indeed, the effects of CQ on preneoplastic and neoplastic lesions are controversial. A previous similar study described that CQ (50 mg/kg, four doses) starting 2 weeks after DEN and 2-acetylaminofluorene treatment significantly decreased the area, but not the number, of GST-P-positive live nodules in rats; these changes were associated with the strong inhibition of cell proliferation activity (Kowalik et al., 2016). Inhibition of hepatocyte cell proliferation and cell cycle progression by CQ was also demonstrated in mice (Lin et al., 2015). However, CQ significantly increased tumor incidence in DEN-treated mice with hyperthyroidism through an increase in the hepatocytes positive for DEN-increased γH2AX (a DNA damage marker) by the inhibition of autophagy (Chi et al., 2016). Therefore, as the effects of CQ on autophagy could be dependent on study conditions in vivo, careful examination and interpretation are required to understand the role of autophagy when treating with an autophagy mediator.

Accumulating evidence suggests that the liver relies strongly on autophagy for its normal function and to prevent the development of disease (Mizushima and Komatsu, 2011). However, the role of autophagy is complicated because autophagy suppresses tumor formation by preventing genomic instability, promoting cell senescence, and limiting inflammation, while after transformation of tumor cells autophagy promotes progression by satisfying the high metabolic demands of the tumor cells and aiding their survival (Czaja et al., 2013). The present study demonstrates that the autophagy inducer, AM, can reduce the number of preneoplastic liver foci via autophagy induction, since the results could be plausibly dependent on DEN-transformed hepatocytes in the preneoplastic lesions. Given the study limitation concerning the use of autophagosome markers in immunohistochemistry, further studies designed to detect autophagy using other autophagosome markers are required to better understand autophagy flux in focal pathological lesions, such as preneoplastic lesions, and carcinoma in situ.

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Conflict of interest—The authors declare that there is no conflict of interest.

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Autophagosome markers in preneoplastic liver foci


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