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Research Article

Cancer Prevention Research

Acyclic Retinoid Inhibits Diethylnitrosamine-Induced Liver Tumorigenesis in Obese and Diabetic C57BLKS/J- +Lepr^{db}/+Lepr^{db} Mice

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Abstract

Obesity and the related metabolic abnormalities are associated with increased risk of hepatocellular carcinoma (HCC). Malfunctioning of retinoid X receptor (RXR) α due to phosphorylation by Ras/MAPK also plays a critical role in liver carcinogenesis. In the present study, we examined the effects of acyclic retinoid (ACR), which targets RXRα, on the development of diethylnitrosamine (DEN)-induced liver tumorigenesis in C57BLKS/J- +Lepr^{db}/+Lepr^{db} (*db/db*) obese mice. Male *db/db* mice were given tap water containing 40 ppm DEN for 2 weeks, after which they were fed a diet containing 0.03% or 0.06% of ACR throughout the experiment. In mice treated with either dose of ACR for 34 weeks, the development of liver cell adenomas was significantly inhibited as compared with basal diet-fed mice. ACR markedly inhibited the activation of Ras and phosphorylation of the ERK (extracellular signal-regulated kinase) and RXRα proteins in the livers of experimental mice. It also increased the expression of *RARβ* and *p21^{CIP1}* mRNA while decreasing the expression of *cyclin D1*, *c-Fos*, and *c-Jun* mRNA in the liver, thereby restoring RXRα function. Administration of ACR improved liver steatosis and activated the AMPK protein. The serum levels of insulin decreased by ACR treatment, whereas the quantitative insulin sensitivity check index (QUICKI) values increased, indicating improved insulin sensitivity. The serum levels of TNF-α and the expression levels of *TNF-α*, *IL-6*, and *IL-1β* mRNA in the livers of DEN-treated *db/db* mice were decreased by ACR treatment, suggesting attenuation of the chronic inflammation induced by excessive fatty deposits. ACR may be, therefore, useful in the chemoprevention of obesity-related HCC. *Cancer Prev Res; 4(1); 128–36. ©2010 AACR.*

Introduction

Hepatocellular carcinoma (HCC) is a serious health-care problem worldwide. The risk factors associated with the development of HCC include chronic hepatitis B and/or hepatitis C infection, particularly with subsequent cirrhosis. Recent evidence also indicates that obesity and the related metabolic abnormalities, especially diabetes mellitus, increase the risk of HCC (1–3). In a rodent model, the occurrence of diethylnitrosamine (DEN)-induced liver tumorigenesis was found to be significantly higher in obese and diabetic C57BLKS/J-+Lepr^{db}/+Lepr^{db} (*db/db*) mice than in genetic control mice (4). Diabetes mellitus has been shown to increase the risk of primary HCC in patients with viral hepatitis (5). Insulin resistance is also significantly associated with the recurrence of stage I HCC after curative treatment (6). Nonalcoholic fatty liver disease (NAFLD) is a hepatic manifestation of the insulin resistance syndrome, and in a subset of NAFLD patients, the condition progresses to nonalcoholic steatohepatitis, which involves severe inflammation and therefore poses the threat of HCC (7, 8). Coexistent obesity or steatosis exacerbates liver injury and fibrosis and thus is involved in liver tumorigenesis (9). Therefore, patients with obesity and insulin resistance comprise a high-risk group for HCC, and their treatment must target the prevention of this malignancy.

Acyclic retinoid (ACR, the same substance as NIK-333), a synthetic retinoid, apparently exerts chemopreventive effects on the development of HCC (10). It inhibits experimental liver carcinogenesis and suppresses the

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growth of HCC-derived cells by inducing apoptosis and causing cell-cycle arrest in G_0–G_1 (11–15). These effects of ACR are associated with its agonistic activity for distinct nuclear retinoid receptors—retinoid X receptors (RXR) and retinoic acid receptors (RAR), both of which have 3 subtypes (α, β, and γ; 16)—and subsequent expression of the ACR target genes *RAR*β and $p21^{CIP1}$ (12–15). A clinical trial revealed that oral administration of ACR significantly reduced the incidence of postntherapeutic HCC recurrence and improved the survival rates of patients (17, 18). A phase II/III trial of ACR confirmed its effectiveness in preventing second primary HCC in hepatitis C virus–positive patients in a large-scale (n = 401) randomized, placebo-controlled trial; hazard ratio for recurrence-free survival with ACR 600 mg/d versus placebo was 0.27 (95% CI, 0.07–0.96) after 2 years randomization (19).

Among the retinoid receptors, RXRα is considered as one of the most important receptors with respect to the regulation of fundamental cell activities because it forms a heterodimer with other nuclear receptors and thereby acts as the master regulator of nuclear receptors (20). Recent studies indicate that phosphorylation of RXRα abolishes its ability to form a heterodimer with RARβ, and the accumulation of phosphorylated RXRα (p-RXRα, i.e., nonfunctional RXRα), which is caused by activation of the Ras/mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) signaling pathway, plays a critical role in the development of HCC (10, 21, 22). On the other hand, the effects of ACR in suppressing growth and inducing apoptosis in HCC cells depend on the inactivation of Ras-ERK signaling system and subsequent RXRα dephosphorylation (15, 23, 24). In the present study, we examined the effects of ACR on obesity-related liver tumorigenesis by focusing on the inhibition of RXRα phosphorylation. We also examined whether ACR treatment improves the insulin resistance, liver steatosis, and inflammatory condition caused by obesity with DEN-treated *db/db* mice, a useful preclinical model, to evaluate the mechanisms underlying the inhibition of obesity-related liver tumorigenesis by chemopreventive drugs (4).

Materials and Methods

Animals and chemicals

Four-week-old male *db/db* mice were obtained from Japan SLC, Inc. All mice received humane care and were housed at Gifu University Life Science Research Center in accordance with the Institutional Animal Care Guidelines. DEN was purchased from Sigma Chemical Co. ACR was supplied by Kowa Pharmaceutical Co.

Experimental procedure

The experimental protocol, which was approved by the Institutional Committee of Animal Experiments of Gifu University, was as described previously (4). At 5 weeks of age, 40 *db/db* mice were randomly divided into 5 groups. All the mice in groups 1 (n = 10), 2 (n = 10), and 3 (n = 10) were given tap water containing 40 ppm of DEN for the first 2 weeks, which is sufficient to develop liver neoplasms in *db/db* mice (4). After DEN treatment, the mice in groups 2 and 3 were fed the basal diet CRF-1 (Oriental Yeast Co.) containing 0.03% ACR (group 2) or 0.06% ACR (group 3), respectively, with free access to the feed till the end of experiment. Group 4 (n = 5) was fed the CRF-1 diet containing 0.06% ACR. The mice in groups 1 and 5 (n = 5) were fed the CRF-1 diet throughout the experiment. The rationale for the doses (0.03% and 0.06%) selection of ACR was based on previous studies, in which similar doses of ACR inhibited experimental liver carcinogenesis induced by chemical agents (25, 26). At 41 weeks of age (after 34 weeks of ACR treatment), all the mice were sacrificed by CO_2 asphyxiation to check for the development of HCC, liver cell adenoma, and foci of cellular alteration (FCA).

Histopathologic analysis

At sacrifice, the livers were immediately removed and macroscopically inspected for the presence of neoplasms. Maximum sagittal sections of each lobe (6 lobes) were used for histopathologic examination. For all experimental groups, 4-μm thick sections of formalin-fixed, paraffin-embedded livers were stained routinely with hematoxylin and eosin (H&E) for histopathologic examination. The presence of HCC, liver cell adenoma, and FCA was judged according to previously described criteria (27). The multiplicity of FCA was assessed on a per unit area (cm^2) basis.

Ras activation assay

Ras activity was determined using a Ras activation assay kit (Upstate Biotechnology) according to the manufacturer's instructions. Ras was precipitated in equivalent amounts of liver extract (50 μg) from DEN-treated mice (groups 1–3) by using Raf-1/Ras-binding domain-immobilized agarose, which was then subjected to Western blot analysis using anti-Ras antibody (24). The intensity of the blots was quantified using NIH imaging software Version 1.62.

Protein extraction and Western blot analysis

Total protein was extracted from the nontumor site of livers of DEN-treated mice, and equivalent amounts of proteins (30 μg per lane) were examined by Western blot analysis (4). Previously described primary antibodies for RXRα (ΔN-197 and D-20), ERK, phosphorylated ERK (p-ERK), Stat3, p-Stat3, AMP-activated kinase (AMPK), p-AMPK, and GAPDH were used (15, 22, 28, 29). The ΔN-197 antibody is considered a specific antibody for the p-RXRα protein (22, 23). The GAPDH antibody served as a loading control.

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RNA extraction and quantitative real-time reverse transcription PCR

Total RNA was isolated from the nontumor site livers of DEN-treated mice by using the RNAqueous-4PCR kit (Ambion Applied Biosystems). cDNA was amplified from 0.2 μg of total RNA by using the SuperScript III First-Strand Synthesis System (Invitrogen), and quantitative real-time reverse transcription PCR (RT-PCR) analysis was carried out as described previously (4). The specific primers used for amplification of the *TNF-α*, *IL-6*, *IL-1β*, and *β-actin* genes were as described previously (30). The primers for the amplification of *RARβ*, *p21^{CIP1}*, *cyclin D1*, *c-Jun*, and *c-Fos* genes are listed in Supplementary Table S1.

Clinical chemistry

Before sacrifice, the mice were fasted for 6 hours, and at sacrifice, blood samples were collected for assaying the serum concentrations of insulin, glucose, and TNF-α, which was as described previously (4, 29). The serum TNF-α (Shibayagi) levels were determined using an enzyme immunoassay according to the manufacturer's protocol. Insulin resistance was estimated by determining the quantitative insulin sensitivity check index (QUICKI) as follows: QUICKI = $1/[\log(I_0) + \log(G_0)]$, where I_0 is the fasting insulin level and G_0 is the fasting glucose level, which correlates with the glucose clamp method (31).

Hepatic lipid analysis

Approximately 200 mg of frozen liver was homogenized, and lipids were extracted using Folch's method (32). The levels of triglyceride in the liver were measured using the triglyceride E-test kit (Wako Pure Chemical Co.) according to the manufacturer's protocol. To visualize the intrahepatic lipids, Sudan III staining was conducted using the standard procedure with frozen sections.

Statistical analysis

The results are presented as the mean ± SD and were analyzed using the GraphPad Instat software program Version 3.05 (GraphPad Software) for Macintosh. Differences among the groups were analyzed by either 1-way ANOVA or, as required, by 2-way ANOVA. When the ANOVA showed a statistically significant effect ($P < 0.05$), each experimental group was compared with the control group by using the Tukey–Kramer multiple comparisons test. The differences were considered significant when the 2-sided P value was less than 0.05.

Results

General observations

As shown in Table 1, no significant differences were observed in the body, kidney, and fat weights among the groups at the end of the study. A significant decrease in the liver weight was observed in the ACR-treated groups as compared with the basal diet-fed group ($P < 0.05$ or $P < 0.01$), irrespective of DEN treatment. Histopathologic examination showed the absence of ACR toxicity in the liver, kidney, and spleen (data not shown).

Effects of ACR on DEN-induced liver tumorigenesis in *db/db* mice

Table 2 summarizes the incidence and multiplicity of liver neoplasms (adenoma and HCC) and FCA in the mice from all groups. FCA developed in the livers of mice from all groups, irrespective of DEN treatment. On the other hand, liver cell adenomas developed only in the DEN-treated *db/db* mice. HCCs also developed in all DEN-treated groups; however, the incidence (10% in each group) wan not high. These findings might be associated with experimental protocol because the duration of the experiments (41 weeks) was sufficient to develop adenoma but not HCC. In mice treated with either dose (0.03% and 0.06%) of ACR, the incidence ($P < 0.01$ in each comparison) and multiplicity of adenoma ($P < 0.05$ or $P < 0.01$) were significantly inhibited compared to ACR-untreated mice. The number of FCA was also significantly decreased by ACR treatment, irrespective of DEN treatment ($P < 0.001$ or $P < 0.05$).

Effects of ACR on Ras activity and phosphorylation of RXRα, ERK, and Stat3 proteins in the livers of DEN-treated *db/db* mice

ACR prevents the growth of HCC cells by inactivating Ras-ERK and dephosphorylating RXRα, thereby restoring RXRα function (10, 15, 23, 24). Stat3 is also an ACR target for the inhibition of cancer cell growth (28). Therefore, the effects of ACR on the inhibition of Ras activity and phosphorylation of the RXRα, ERK, and Stat3 proteins were examined in this study by using an obesity-related liver tumorigenesis model. As shown in Figure 1A, the activity of Raf-1–bound Ras in the liver was significantly inhibited by treatment with either dose of ACR ($P < 0.01$). The expression levels of the p-ERK and p-RXRα proteins were also decreased by ACR treatment (Fig. 1B), indicating that ACR inhibits the development of obesity-related liver neoplasms, at least in part, by dephosphorylating RXRα and thereby restoring its function. At both doses, ACR also decreased the expression levels of the p-Stat3 protein in the livers of DEN-treated *db/db* mice (Fig. 1B).

Effects of ACR on the expression levels of RARβ, p21^{CIP1}, cyclin D1, c-Fos, and c-Jun mRNA in the livers of DEN-treated *db/db* mice

ACR inhibits the growth of HCC cells by increasing the cellular levels of RARβ and p21^{CIP1} but decreasing the levels of cyclin D1, and these effects might be associated with the restoration of RXRα function (12–15). It also suppresses the growth of cancer cells by inhibiting the activity of AP-1, which comprises the Jun and Fos oncoprotein families (28). Therefore, the effect of ACR on the mRNA levels of these molecules was examined next. As shown in Figure 1C, quantitative real-time RT-PCR analysis indicated that ACR treatment

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Table 1. Body, liver, kidney, and fat weights of the experimental mice

Group no.	Treatment	No. of mice	Weight, g			
			Body	Liver	Kidney	Fat[a]
1	DEN alone	10	71.2 ± 8.8[b]	4.5 ± 0.8	0.9 ± 1.0	7.5 ± 2.2
2	DEN + 0.03% ACR	10	65.7 ± 7.2	3.3 ± 1.1[c]	0.5 ± 0.1	6.0 ± 1.5
3	DEN + 0.06% ACR	10	66.0 ± 7.4	3.0 ± 0.7[d]	0.5 ± 0.1	5.7 ± 1.3
4	0.06% ACR alone	5	66.0 ± 7.4	3.0 ± 0.7[e]	0.5 ± 0.1	5.7 ± 1.3
5	Basal diet	5	67.9 ± 7.8	4.8 ± 1.0	0.6 ± 0.1	6.2 ± 1.4

[a]White adipose tissue of the periorchis and retroperitoneum.
[b]Mean ± SD.
[c]Significantly different from group 1 by Tukey–Kramer multiple comparison test ($P < 0.05$).
[d]Significantly different from group 1 by Tukey–Kramer multiple comparison test ($P < 0.01$).
[e]Significantly different from group 5 by Tukey–Kramer multiple comparison test ($P < 0.05$).

significantly increased the expression levels of *RARβ* and $p21^{CIP1}$ mRNA, especially *RARβ* mRNA, in the livers of DEN-exposed *db/db* mice ($P < 0.01$). On the other hand, the expression levels of *cyclin D1*, *c-Fos*, and *c-Jun* mRNA were significantly decreased by ACR treatment ($P < 0.01$).

Effects of ACR on hepatic steatosis and the activation of AMPK in the livers of DEN-treated *db/db* mice

Hepatic steatosis is considered a promoter of the development of HCC (8, 9). Therefore, whether ACR treatment enhances the accumulation of lipids in the liver of experimental mice was examined. Examination of Sudan III–stained sections revealed that ACR treatment significantly improved macrovesicular steatosis in the livers of DEN-treated *db/db* mice (Fig. 2A, top panels). The triglyceride levels in the liver were also significantly decreased in mice treated with ACR at either dose ($P < 0.05$) in comparison with those fed the basal diet (Fig. 2A, bottom graph). Moreover, ACR markedly phosphorylated (activated) the AMPK protein, which is a critical serine/threonine kinase that monitors cellular energy status (33), in the livers of the experimental mice (Fig. 2B).

Effects of ACR on insulin resistance in DEN-treated *db/db* mice

Insulin resistance plays a critical role in the development of HCC (1–6). Therefore, the effects of ACR on the levels of serum insulin and QUICKI values, which indicate the degree of insulin sensitivity, were examined in DEN-treated *db/db* mice. As shown in Figure 2C, the serum insulin level was decreased ($P < 0.05$) whereas the QUICKI value was increased in mice treated with 0.06% ACR ($P < 0.05$)

Table 2. Incidence and multiplicity of hepatic neoplasms and FCA in the experimental mice

Group no.	Treatment	No. of mice	Incidence		Multiplicity[a]		FCA
			Adenoma	HCC	Adenoma	HCC	(No./cm^2)
1	DEN alone	10	7/10 (70%)	1/10 (10%)	1.3 ± 1.2[b]	0.1 ± 0.3	15.1 ± 3.5[c]
2	DEN + 0.03% ACR	10	1/10 (10%)[e]	1/10 (10%)	0.2 ± 0.6[e]	0.1 ± 0.3	6.6 ± 2.5[f]
3	DEN + 0.06% ACR	10	1/10 (10%)[e]	1/10 (10%)	0.1 ± 0.3[g]	0.1 ± 0.3	2.8 ± 1.8[f]
4	0.06% ACR alone	5	0/5 (0%)	0/5 (0%)	0	0	3.0 ± 2.8[h]
5	Basal diet	5	0/5 (0%)	0/5 (0%)	0	0	8.0 ± 1.2

[a]Number of neoplasms per mouse.
[b]Mean ± SD.
[c]Significantly different from group 5 by Tukey–Kramer multiple comparison test ($P < 0.001$).
[d]Significantly different from group 1 by Fisher's exact probability test ($P < 0.01$).
[e]Significantly different from group 1 by Tukey–Kramer multiple comparison test ($P < 0.05$).
[f]Significantly different from group 1 by Tukey–Kramer multiple comparison test ($P < 0.001$).
[g]Significantly different from group 1 by Tukey–Kramer multiple comparison test ($P < 0.01$).
[h]Significantly different from group 5 by Tukey–Kramer multiple comparison test ($P < 0.05$).

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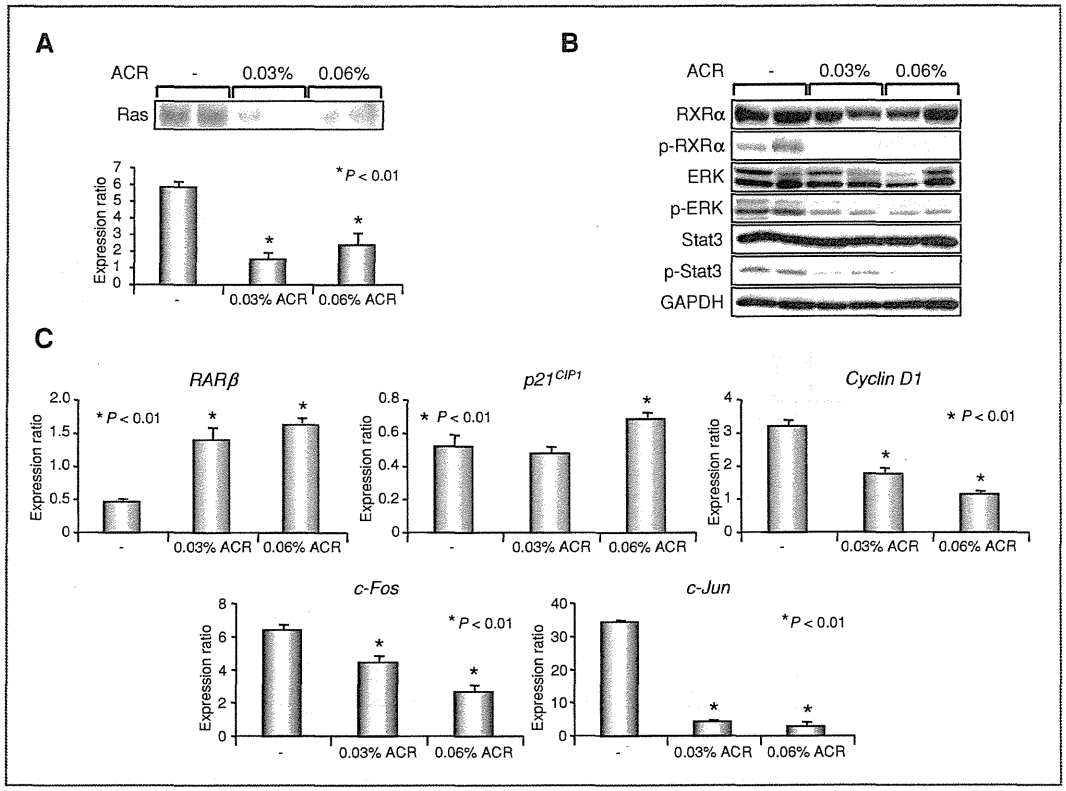

Figure 1. Effects of ACR on Ras activity; phosphorylation of RXRα, ERK, and Stat3 proteins; and the expression of target genes in the livers of DEN-treated *db/db* mice. The total proteins and mRNAs were extracted from the livers of DEN-treated mice. A, the Ras activities were determined using a Ras activation assay kit (top). The relative intensity of the blots was quantified by densitometry and is displayed in the bottom graph. B, the expression levels of the RXRα, p-RXRα, ERK, p-ERK, Stat3, and p-Stat3 proteins were examined by Western blot analysis, using the respective antibodies. Equal protein loading was verified by the detection of GAPDH. Two lanes represent protein samples from two different mice from each group. Repeat Western blots yielded similar results. C, the expression levels of *RARβ*, *p21*^{CIP1}, *cyclin D1*, *c-Fos*, and *c-Jun* mRNA were examined by quantitative real-time RT-PCR using specific primers. β-Actin was used as a control. Each experiment was performed in triplicate, and the average value was calculated. Values are the mean ± SD. *, $P < 0.01$ vs. ACR-untreated group.

compared with those in the basal diet–fed group. These findings suggest that ACR improves insulin resistance in obese and diabetic *db/db* mice.

Effects of ACR on the serum levels of TNF-α and hepatic expression of TNF-α, IL-6, and IL-1β mRNA in DEN-treated *db/db* mice

Because a state of chronic inflammation induced by excessive production of storage lipids and insulin resistance is associated with obesity-related liver carcinogenesis (34), the effects of ACR on the levels of the proinflammatory cytokines TNF-α, IL-6, and IL-1β in DEN-treated *db/db* mice were examined. As shown in Figure 3A, the serum levels of TNF-α were decreased after ACR treatment ($P < 0.01$). Furthermore, the expression levels of *TNF-α*, *IL-6*, and *IL-1β* mRNA in the livers of DEN-treated *db/db* mice were also significantly decreased by ACR treatment ($P < 0.01$). The decrease was most apparent in the levels of *IL-6* mRNA: the inhibition rates were about 85% at both doses of ACR (Fig. 3B).

Discussion

In the present health care scenario, the effects of obesity, including the promotion of cancer, are critical issues that need to be resolved and HCC is one of the representative malignancies influenced by excessive body weight and related metabolic abnormalities (1–3, 5, 6). A recent clinical trial revealed that supplementation of food with branched-chain amino acids (BCAA), which improves insulin resistance (35), reduced the risk of HCC in obese patients with chronic viral liver disease (3). BCAA supplementation also suppresses liver tumorigenesis in obese and diabetic *db/db* mice by improving insulin resistance and attenuating liver steatosis and fibrosis (4). The results of the present study clearly indicated that ACR also effectively

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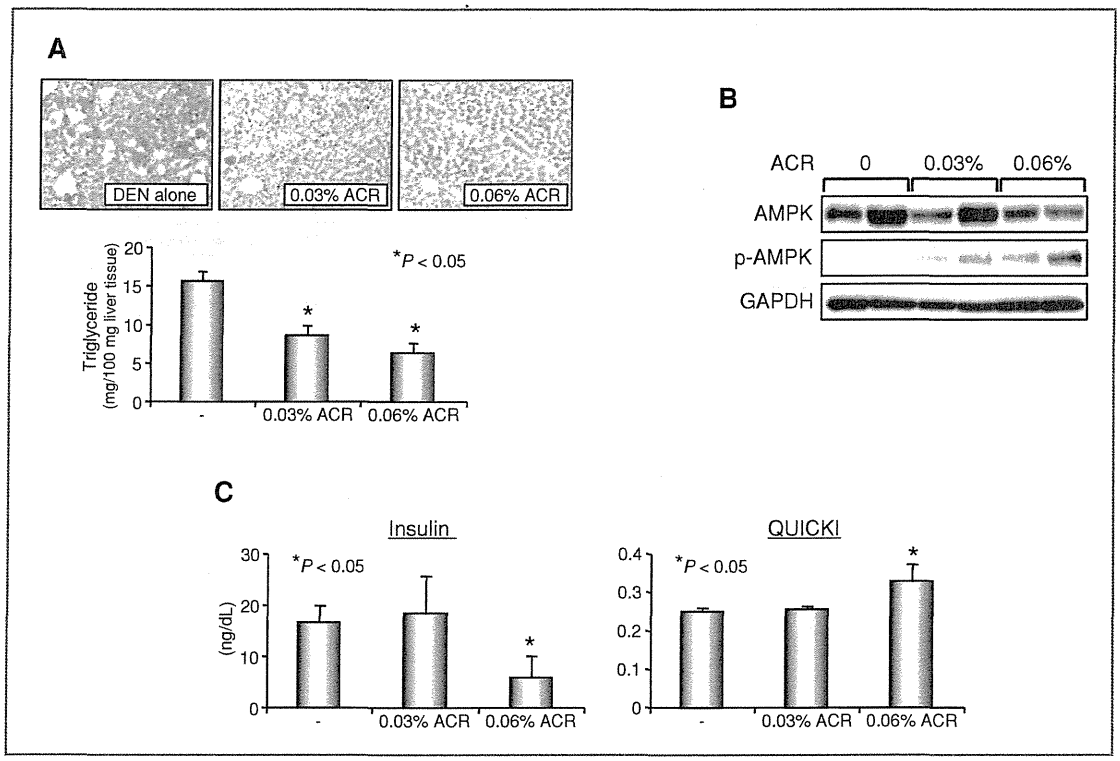

Figure 2. Effects of ACR on hepatic steatosis, the activation of the AMPK protein in the liver, and the levels of serum insulin and insulin sensitivity in DEN-treated *db/db* mice. A, frozen liver sections from DEN-exposed mice treated with or without ACR were stained with Sudan III to show steatosis (top). Hepatic lipids were extracted from the frozen livers of these mice, and the triglyceride levels were measured (bottom). B, the total proteins were extracted from the livers of DEN-treated mice, and the expression levels of the AMPK and p-AMPK proteins were examined by Western blot analysis, using the respective antibodies. A GAPDH antibody served as a loading control. C, the serum concentration of insulin was measured by enzyme immunoassay (left). The QUICKI value was calculated to evaluate insulin sensitivity (right). Values are the mean ± SD. *, $P < 0.05$ vs. ACR-untreated group.

prevents the development of obesity-related liver cell adenomas, and these effects are associated with improvement of hepatic steatosis and insulin resistance. Therefore, the findings of the present study, together with the results of previous studies using BCAA (3, 4), suggest that improvement of metabolic abnormalities by pharmaceutical or nutritional intervention might be an effective strategy for inhibiting obesity-related liver tumorigenesis.

Several biological effects of ACR are relevant to the prevention of obesity-related hepatotumorigenesis. First, it should be noted that ACR inhibits RXRα phosphorylation by suppressing the Ras/ERK signaling pathway in the livers of DEN-treated *db/db* mice. These findings are consistent with those of previous *in vitro* studies (15, 23, 24), but this is the first *in vivo* experiment, and the results seem to be significant because RXRα malfunction due to the phosphorylation by Ras-ERK plays a role in liver carcinogenesis and phosphorylated RXRα is therefore a critical target for HCC chemoprevention (10, 21). ACR suppresses the growth of HCC cells by inhibiting RXRα phosphorylation and restoring its original function as a master regulator of nuclear receptors (15, 22–24). Therefore, the expression levels of the *RARβ*, *p21^{CIP1}*, *cyclin D1*, *c-Fos*, and *c-Jun* genes, which are ACR targets (12–15, 28), were notably regulated by treatment with this agent. Among these molecules, RARβ seems to be the most important with respect to the induction of apoptosis (36). The upregulation of p21^{CIP1}, which negatively modulates cell-cycle progression, also activates the promoter region of the *RARβ* gene (37). Because RARβ can form a heterodimer with RXRα and thus synergistically inhibit the growth of HCC cells (14, 15), its induction might also have played a role in preventing the development of liver tumors in the present study. In addition, p21^{CIP1} induction, which might be caused by activation of transforming growth factor (TGF)-β, also contributes to prevent the development of liver neoplasms because TGF-β induces senescence and inhibits growth in HCC cells by upregulating p21^{CIP1} and ACR can activate latent TGF-β in liver stellate cells (38, 39).

Next, the effects of ACR in improving hepatic steatosis and insulin resistance, both of which accelerate HCC development (7–9), are discussed. These effects might also

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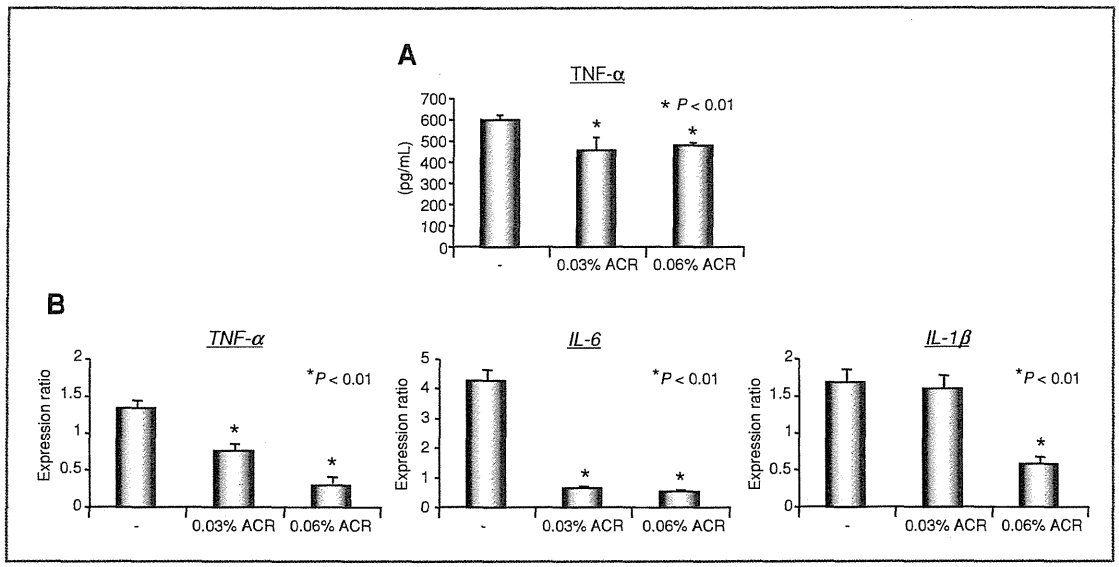

Figure 3. Effects of ACR on the serum levels of TNF-α and the expression levels of *TNF-α*, *IL-6*, and *IL-1β* mRNA in the livers of DEN-treated *db/db* mice. A, the serum concentration of TNF-α was measured by enzyme immunoassay. B, the expression levels of *TNF-α*, *IL-6*, and *IL-1β* mRNA were examined by quantitative real-time RT-PCR using specific primers. The expression levels of these mRNAs were normalized to the level of the β-*actin* mRNA. Values are the mean ± SD. *, $P < 0.01$ vs. ACR-untreated group.

be associated with RXRα dephosphorylation, as RXR can control insulin sensitization and lipid metabolism by forming a heterodimer with peroxisome proliferator-activated receptor (PPAR), an important molecule in the regulation of lipid homeostasis and energy metabolism (40). This speculation is interesting because the inhibition of RXRα phosphorylation and the activation of the RXR/PPAR heterodimer are also activities that cooperatively inhibit the growth of cancer cells (41). In addition, ACR might improve these metabolic abnormalities by activating AMPK, which increases glucose uptake and fatty acid oxidation but decreases fatty acid synthesis (33). This is another positive finding with regard to the prevention of hepatotumorigenesis because decreased AMPK activation is implicated in tumor development and therefore may be a promising target for cancer chemoprevention (42, 43). For instance, a human study suggests that metformin, an AMPK activator used to treat type 2 diabetes mellitus, reduces the cancer risk in diabetic patients (44). Dietary energy restriction suppresses mammary tumorigenesis in rats by increasing the levels of activated AMPK (45). Pitavastatin, a lipophilic statin, was found to prevent obesity- and diabetes-related colon carcinogenesis in mice by activating AMPK in the colonic mucosa (29). These reports suggest the possibility that activation of AMPK by ACR aided in suppressing the development of obesity-related liver cells adenomas, as observed in the present study.

Insulin resistance and lipid accumulation in the liver produce inflammatory changes in the liver (7–9). ACR might decrease the serum levels of TNF-α and the expression levels of *TNF-α*, *IL-6*, and *IL-1β* mRNA in the livers of experimental mice by improving hepatic steatosis and insulin resistance. These findings are significant because obesity-related HCC development clearly depends on enhanced production of TNF-α and IL-6, which cause hepatic inflammation and activate ERK and Stat3 (34). TNF-α, which lies at the core of the association between obesity and insulin resistance (46), contributes to obesity-induced IL-6 production and hepatocarcinogenesis (34). IL-6 is a major Stat3 activator in the liver, and the activation of the IL-6–Stat3 axis plays a critical role in HCC development (47, 48). In addition, uncontrolled activation of the Ras/ERK and Jak/Stat pathways is essential for HCC development (49). In the present study, ubiquitous activation of Ras-ERK signaling presumably caused accumulation of the p-RXRα protein in the liver of the obese mice. Our findings indicate that the effects of ACR in improving the inflammatory response and inhibiting Ras-ERK and Stat3 activation are crucial to prevent the development of obesity-related liver tumors.

Finally, it should be emphasized again that prevention of HCC by targeting hepatic steatosis, insulin resistance, and the state of chronic inflammation, which are caused by dysregulation of energy homeostasis, might be one of the promising strategies for the treatment of obese individuals who are at an increased risk of developing HCC (3, 4). ACR seems to be potentially effective and critical candidate for this purpose because it can improve hepatic steatosis and insulin resistance while also attenuating chronic inflammation. It inhibits RXRα phosphorylation induced by

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Ras-ERK activation, which might be associated with excess adipose tissue, and this effect is also important for preventing obesity-related liver tumorigenesis. The findings of the present study, together with the results of previous clinical trials indicating that ACR can significantly prevent the development of HCC in patients with viral cirrhosis without causing serious adverse effects (17–19), encourage the clinical usage of this agent for cirrhotic patients with obesity and diabetes. On the other hand, careful observation is required to apply a retinoid in clinical practice because of its potential toxicity. For instance, ACR may worsen hypertriglyceridemia in obese and diabetic subjects, which is a side effect observed in previous clinical trial (17), limiting the application of ACR to such subjects.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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RESEARCH ARTICLE Open Access

Pitavastatin suppresses diethylnitrosamine-induced liver preneoplasms in male C57BL/KsJ-*db/db* obese mice

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Abstract

Background: Obesity and related metabolic abnormalities, including inflammation and lipid accumulation in the liver, play a role in liver carcinogenesis. Adipocytokine imbalances, such as decreased serum adiponectin levels, are also involved in obesity-related liver tumorigenesis. In the present study, we examined the effects of pitavastatin - a drug used for the treatment of hyperlipidemia - on the development of diethylnitrosamine (DEN)-induced liver preneoplastic lesions in C57BL/KsJ-*db/db* (*db/db*) obese mice.

Methods: Male *db/db* mice were administered tap water containing 40 ppm DEN for 2 weeks and were subsequently fed a diet containing 1 ppm or 10 ppm pitavastatin for 14 weeks.

Results: At sacrifice, feeding with 10 ppm pitavastatin significantly inhibited the development of hepatic premalignant lesions, foci of cellular alteration, as compared to that in the untreated group by inducing apoptosis, but inhibiting cell proliferation. Pitavastatin improved liver steatosis and activated the AMPK-α protein in the liver. It also decreased free fatty acid and aminotransferases levels, while increasing adiponectin levels in the serum. The serum levels of tumor necrosis factor (TNF)-α and the expression of *TNF-α* and *interleukin-6* mRNAs in the liver were decreased by pitavastatin treatment, suggesting attenuation of the chronic inflammation induced by excess fat deposition.

Conclusions: Pitavastatin is effective in inhibiting the early phase of obesity-related liver tumorigenesis and, therefore, may be useful in the chemoprevention of liver cancer in obese individuals.

Background

Hepatocellular carcinoma (HCC) is a serious healthcare problem worldwide because of its increasing morbidity and high mortality. Chronic inflammation of the liver and subsequent cirrhosis, which are highly correlated with hepatitis B and hepatitis C viruses infection and alcoholic liver disease, are the strongest risk factors for HCC development. Recent evidence also indicates that obesity and related metabolic abnormalities, especially diabetes mellitus and insulin resistance, raise the risk of HCC [1-4]. In obese individuals, high levels of free fatty acid (FFA) flux into the liver from excess adipose tissue. This in turn promotes hepatic steatosis and inflammation through the production of pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α and interleukin (IL)-6, and is closely associated with liver carcinogenesis [5-7]. Aberrant lipogenesis in the liver, which is closely linked to obesity and metabolic syndrome, is also a dominant event in liver carcinogenesis and human HCC progression [8]. Non-alcoholic fatty liver disease (NAFLD) is a hepatic manifestation of the metabolic syndrome and a proportion of patients with this disease can progress to non-alcoholic steatohepatitis (NASH), which involves the risk of developing cirrhosis and HCC [9]. Therefore, in addition to lifestyle modification to reduce body weight, active pharmacotherapy is considered to be necessary for the management of NASH. For instance, metformin and thiazolidinediones,

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both of which increase insulin sensitivity, might be useful for the treatment of patients with NASH [10].

Statins, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, are widely used for the treatment of hyperlipidemia and have been shown to reduce the risk of cardiovascular disease [11]. Statins have recently also been suggested to be possible candidates for the management of NASH/NAFLD, which frequently coexist with hyperlipidemia and cardiovascular disease [12]. A pilot study revealed that treatment with atorvastatin decreases TNF-α serum levels and improves biochemical and histological features of disease activity in NASH patients with dyslipidemia [13]. The use of atorvastatin in hyperlipidemic patients complicated with NAFLD also improves serum transaminase levels and prevents hepatic fibrosis progression [14]. In a mice model, pitavastatin, a recently developed lipophilic statin, has been shown to ameliorate severe hepatic steatosis by enhancing hepatic free acid (FA) β-oxidation activity [15].

In addition to the lipid-lowering and anti-inflammatory effects, recent studies have revealed that statins appear to have anticancer and cancer chemopreventive properties [16,17]. A large cohort study showed that statin use is associated with a reduced risk of HCC in patients with diabetes [18]. Statins inhibit cell proliferation and induce apoptosis in human HCC-derived cells [19,20]. In addition, pitavastatin prevents obesity-related colorectal carcinogenesis by correcting adipocytokine imbalance and attenuating colonic inflammation in C57BL/KsJ-*db/db* (*db/db*) mice suffering from obesity and hyperlipidemia [21]. These findings suggest the possibility that long-term use of statins may also be effective for preventing the progression of obesity-related liver tumorigenesis. Our recent study showed that diethylnitrosamine (DEN)-induced liver tumorigenesis is significantly enhanced in *db/db* mice [22]. In the present study, we examined the effects of pitavastatin on the development of DEN-induced hepatic preneoplastic lesions, foci of cellular alteration (FCA), while focusing on the improvement of liver steatosis and inflammation using a *db/db* mice model.

Methods

Animals and chemicals

Four-week-old male *db/db* mice were obtained from Japan SLC Inc. (Shizuoka, Japan) and were humanely maintained at the Gifu University Life Science Research Center in accordance with the Institutional Animal Care Guidelines. DEN was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Pitavastatin was obtained from Kowa Pharmaceutical Co. (Tokyo, Japan).

Experimental procedure

The animal experiment was approved by the Committee of Institutional Animal Experiments of Gifu University [22]. At 5 weeks of age, all 36 mice were administered tap water containing 40 ppm DEN for the first 2 weeks of the experiment. After DEN treatment, Groups 2 (n = 12) and 3 (n = 12) were given a basal diet (CRF-1, Oriental Yeast Co., Tokyo, Japan) containing 1 and 10 ppm pitavastatin, respectively, until the end of the experiment. Group 1 (n = 12) acted as the control and was fed only a basal diet throughout the experiment. At 21 weeks of age (after 14 weeks of pitavastatin treatment), all the mice were sacrificed to analyze the development of FCA. Since neither C57B6 nor C57BL/KsJ-+/+ mice - the genetic controls for *db/db* mice - develop FCA and liver neoplasms by DEN administration during this period [22], control experimentation using these mice was not conducted in the present study.

Histopathology and immunohistochemical analysis for PCNA

Maximum sagittal sections of each lobe (6 sublobes) were used for histopathological examination. For all experimental groups, 4 μm-thick sections of formalin-fixed and paraffin-embedded livers were stained with hematoxylin & eosin (H&E) for histopathology. The presence of FCA, which are phenotypically altered hepatocytes showing swollen and basophilic cytoplasm and hyperchromatic nuclei, was judged according to the criteria described in a previous study [23]. The multiplicity of FCA was assessed on a per unit area (cm^2) basis.

Immunohistochemical staining of proliferating cell nuclear antigen (PCNA), a G_1-to-S phase marker, was performed to estimate the cell proliferative activity of FCA by using an anti-PCNA antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and the labeled streptavidin-biotin method (LSAB kit; DAKO, Glostrup, Denmark) [22]. On the PCNA-immunostained sections, the cells with intensively reacted nuclei were considered to be positive for PCNA, and the indices (%) were calculated in 20 FCA randomly selected from each group.

Protein extraction and western blot analysis

Equivalent amounts of extracted mice liver proteins (20 μg/lane) were examined by western blot analysis [22]. Previously described primary antibodies for AMP-activated kinase-α (AMPK-α), phosphorylated AMPK-α (p-AMPK-α), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used [21], with GAPDH serving as a loading control. The primary antibody for Bad was purchased from Cell Signaling Technology (Beverly, MA, USA). The intensities of the blots were quantified with NIH Image software version 1.62.

RNA extraction and quantitative real-time reverse transcription-PCR

Total RNA was isolated from the livers of experimental mice using the RNAqueous-4PCR kit (Ambion Applied

Biosystems, Austin, TX, USA) and cDNA was amplified from 0.2 μg of total RNA using the SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). Quantitative real-time reverse transcription-PCR (RT-PCR) analysis was performed using specific primers that amplify *TNF-α*, *IL-6*, *Bcl-2*, *Bad*, and *GAPDH* genes, as described previously [21,24].

Clinical chemistry

The blood samples, which were collected at the time of sacrifice after 6 hours of fasting, were used for chemical analyses. The serum TNF-α (Shibayagi, Gunma, Japan), IL-6 (IBL, Gunma, Japan), adiponectin (Otsuka, Tokyo, Japan), and leptin (R&D Systems, Minneapolis, MN, USA) levels were determined by enzyme immunoassay according to the manufacturers' protocol. The serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), free fatty acid (FFA), total cholesterol, and triglyceride were measured with a standard clinical automatic analyzer (type 7180; Hitachi, Tokyo, Japan).

Hepatic lipid analysis

Approximately 200 mg of frozen liver was homogenized, and lipids were extracted using Folch's method [25]. The triglyceride levels in the liver were measured using the triglyceride E-test kit (Wako Pure Chemical Co., Osaka, Japan) [22]. To visualize the intrahepatic lipids, Oil red O staining was utilized based on the standard procedure for frozen liver sections.

Statistical analysis

The results are presented as means ± SD, and were analyzed using the GraphPad Instat software program version 3.05 (GraphPad Software; San Diego, CA) for Macintosh. Differences among the groups were analyzed by either one-way ANOVA or, as required, by two-way ANOVA. When the ANOVA revealed a statistically significant effect ($P < 0.05$), each experimental group was compared with the control group by using the Bonferroni multiple comparisons test. The differences were considered significant when the two-sided P value was < 0.05.

Results

General observations

As presented in Table 1, administration of pitavastatin significantly ($P < 0.01$, Group 1 vs. Groups 2 and 3) and dose dependently ($P < 0.05$, Group 2 vs. Group 3) decreased the value of body mass index (BMI). The body weight and relative weights of liver and white adipose tissue (periorchis and retroperitoneum) of the mice that received 10 ppm pitavastatin were slightly lower than those of the untreated control mice, but the differences were not significant. During the experiment, pitavastatin administration did not cause any clinical symptoms for toxicity. Histopathological examination also revealed the absence of pitavastatin toxicity in the liver, kidney, and spleen (data not shown).

Effects of pitavastatin on DEN-induced liver preneoplastic lesions in db/db *mice*

Liver preneoplastic lesion FCA, which possesses basophilic cytoplasm and hyperchromatic nuclei (Figure 1A), was observed in the livers of mice from all groups at the termination of the experiment. Treatment with a high dose (10 ppm) of pitavastatin significantly inhibited the development of FCA in comparison to both the untreated control mice ($P < 0.001$) and low dose (1 ppm) of pitavastatin-treated mice ($P < 0.05$). Treatment with 1 ppm pitavastatin also demonstrated a tendency to suppress the development of FCA - the inhibition rate being 29% - in comparison to the untreated control mice, but the difference did not reach a statistical significance (Figure 1B).

Effects of pitavastatin on the cellular levels of Bad and Bcl-2 and the proliferation activity in FCA of DEN-treated db/db *mice*

We next examined the effects of pitavastatin on the induction of apoptosis in the liver and the inhibition of cell proliferation in FCA of DEN-treated *db/db* mice. Treatment with both low and high doses of pitavastatin increased the protein levels of Bad, a pro-apoptotic Bcl-2 family member, in the liver of experimental mice (Figure 2A, $P < 0.05$). The mRNA levels of this molecule

Table 1 Body, liver, kidney and white adipose tissue weights of the experimental mice

Group no.	Treatment	No. of mice	Body wt (g)	BMI[a]	Relative wt (g/100 g body wt) of:		
					Liver	Kidney	Fat[b]
1	DEN alone	12	63.1 ± 7.0[c]	7.2 ± 0.6	6.4 ± 1.5	0.9 ± 0.1	9.3 ± 1.0
2	DEN + 1 ppm Pitavastatin	12	59.7 ± 3.9	6.7 ± 0.4[d]	6.0 ± 0.8	0.9 ± 0.1	9.1 ± 0.8
3	DEN + 10 ppm Pitavastatin	12	55.2 ± 9.5	6.2 ± 0.6[d,e]	5.7 ± 1.2	1.0 ± 0.2	8.7 ± 1.0

[a]Body mass index.

[b]White adipose tissue of the periorchis and retroperitoneum.

[c]Mean ± SD.

[d]Significantly different from Group 1 ($P < 0.01$).

[e]Significantly different from Group 2 ($P < 0.05$).

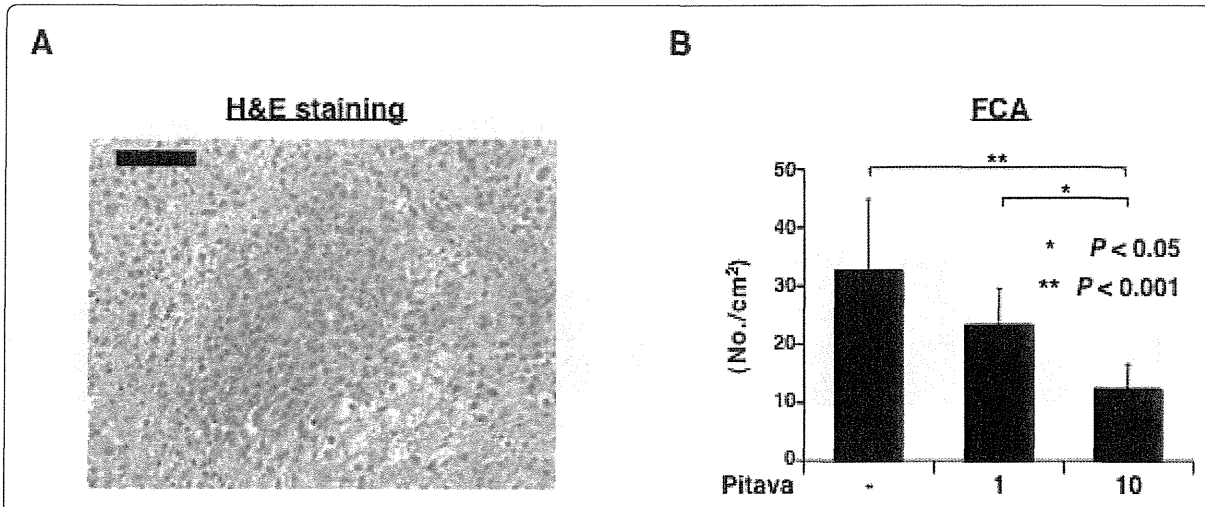

Figure 1 Effects of pitavastatin on DEN-induced FCA in *db/db* mice. (*A*) A representative photograph of FCA induced by DEN in *db/db* mice (H&E staining). Scale bar, 100 μm. (*B*) Average number of FCA in all groups (pitavastatin-untreated, 1 ppm pitavastatin-treated, and 10 ppm pitavastatin-treated groups). Each column represents the mean ± SD. * $P < 0.05$ vs. 1 ppm pitavastatin-treated group and ** $P < 0.001$ vs. the untreated group, respectively.

were also increased by 1 ppm pitavastatin administration (Figure 2B, $P < 0.05$). On the other hand, pitavastatin treatment induced a marked decrease in the levels of an anti-apoptotic molecule Bcl-2 mRNA (Figure 2B, $P < 0.05$). In addition, as shown in Figure 2C, the mean PCNA-labeling indices for FCA in mice treated with 1 ppm (23.9 ± 7.7%) and 10 ppm (16.6 ± 4.0%) pitavastatin were significantly lower than that in the mice which received only DEN (47.7 ± 11.0%; $P < 0.001$ for each comparison). These findings indicate that pitavastatin significantly suppresses FCA, at least in part, by inducing apoptosis and by reducing cell proliferation.

Effects of pitavastatin on hepatic steatosis, activation of AMPK-α protein in the liver, and serum levels of FFA, total cholesterol, and triglyceride in DEN-treated db/db *mice*

Accumulation of lipids in the liver, which is caused by dyslipidemia, is considered to play a role in liver tumorigenesis [5,6]. Therefore, we examined whether pitavastatin improved hepatic steatosis and hyperlipidemia in the experimental mice. Examination of Oil red O stained sections revealed severe hepatic steatosis in the DEN-treated *db/db* mice; however, the mice's conditions were markedly improved by pitavastatin administration (Figure 3A, upper panels). Similar to the histological findings, the levels of intrahepatic triglyceride were also significantly reduced by administration of pitavastatin (Figure 3A, lower panel, $P < 0.001$). Western blot analysis demonstrated that pitavastatin significantly phosphorylated (*i.e.*, activated) AMPK-α - a critical kinase that monitors cellular energy status [26] - in the livers of the experimental mice (Figure 3B, $P < 0.05$). In addition, treatment with both low ($P < 0.01$) and high ($P < 0.001$) doses of pitavastatin decreased the serum levels of FFA, while the levels of total cholesterol and triglyceride were not affected by administration of this agent (Figure 3C).

Effects of pitavastatin on serum levels of AST, ALT, adiponectin, and leptin in DEN-treated db/db *mice*

The serum levels of AST, ALT, adiponectin, and leptin in the experimental mice are listed in Table 2. The elevated serum AST and ALT levels, which might increase due to severe steatosis (Figure 3A), were significantly decreased by treatment with both low ($P < 0.001$) and high ($P < 0.05$) doses of pitavastatin. The serum leptin levels after pitavastatin administration demonstrated a downward trend, but the differences were not significant. However, treatment with this agent markedly increased the serum levels of adiponectin when compared to the control mice ($P < 0.05$).

Effects of pitavastatin on serum TNF-α levels and hepatic expression of TNF-α *and* IL-6 *mRNAs in DEN-treated* db/db *mice*

Chronic inflammation induced by excessive production of storage lipids is closely associated with obesity-related liver carcinogenesis [5-7]. Therefore, the effects of pitavastatin on the serum levels of TNF-α, a central mediator of chronic inflammatory disease, and on the expression of *TNF-α* and *IL-6* mRNAs in the liver of DEN-treated *db/db* mice were examined. Administration

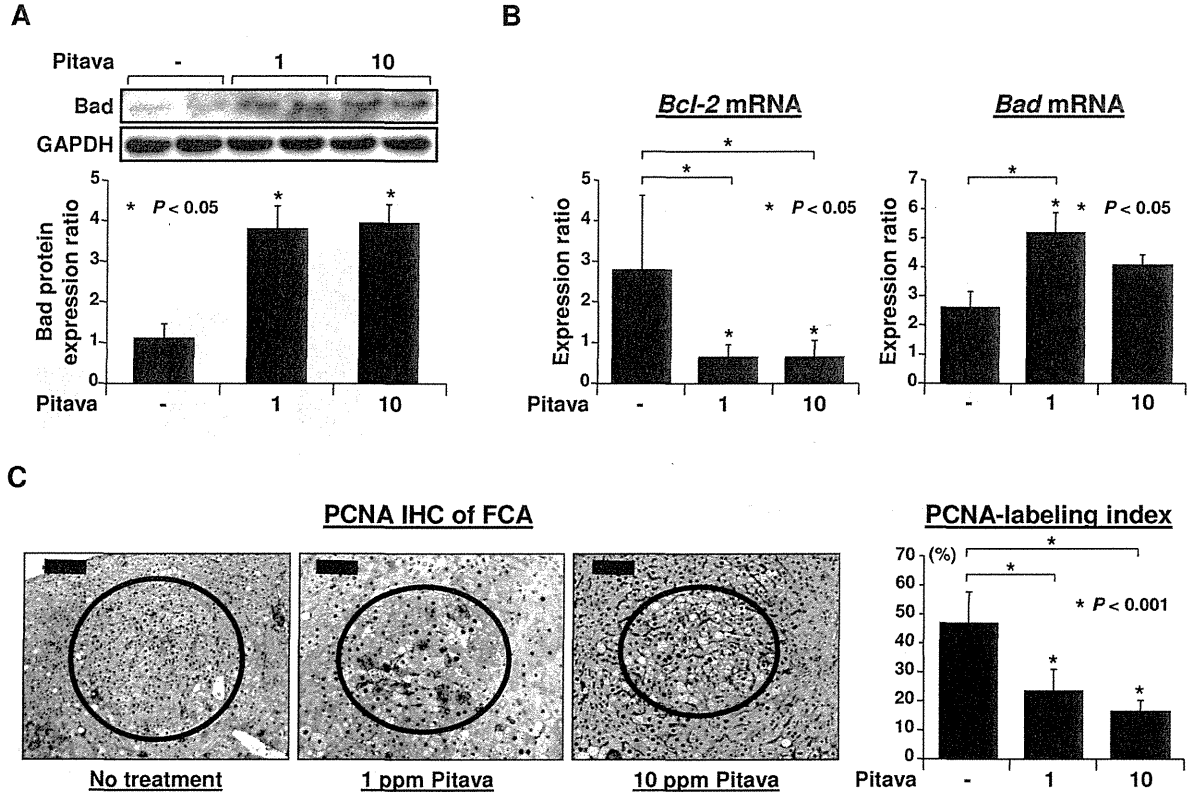

Figure 2 Effects of pitavastatin on the expression levels of Bad and Bcl-2 in the liver and cell proliferation in FCA induced by DEN in *db/db* mice. (*A*) The total proteins were extracted from the livers of experimental mice and the expression levels of the Bad protein were examined by western blot analysis (upper panel). The intensities of the blots were quantified by densitometry (lower panel). GAPDH antibody served as a loading control. Two lanes represent protein samples obtained from 2 different mice in each group. Repeat western blots yielded similar results. Values are the means ± SD. * $P < 0.05$ vs. the untreated group. (*B*) The total RNAs were isolated from the livers of experimental mice and the expression levels of *Bcl-2* and *Bad* mRNAs were examined by quantitative real-time RT-PCR using specific primers. The expression levels of each mRNA were normalized to the level of *GAPDH* mRNA. Values represent the means ± SD. * $P < 0.05$ vs. the untreated group. (*C*) Representative pictures of the PCNA-immunohistochemical analysis of FCA (circled) developed in the livers of Groups 1, 2, and 3 (left panels). The PCNA-labeling indices of FCA developed in the livers of Groups 1 through 3 were determined by counting the PCNA-positive nuclei in FCA (right panel). Scale bars, 200 μm (no treatment) and 100 μm (1 and 10 ppm pitavastatin). * $P < 0.001$ vs. the untreated group.

of both doses of pitavastatin significantly decreased serum TNF-α levels (Figure 4A, $P < 0.05$). Further, quantitative real-time RT-PCR revealed that the expression levels of *TNF-α* and *IL-6* mRNAs in the livers of experimental mice were also significantly decreased after pitavastatin treatment (Figure 4B, $P < 0.05$, respectively), suggesting that pitavastatin attenuated liver inflammation in obese *db/db* mice.

Discussion and Conclusions

Statins lessen hyperlipidemia by competitively inhibiting HMG-CoA reductase, and thus, they are effective in preventing cardiovascular disease [11]. On the other hand, many studies have shown the anticancer and cancer chemopreventive effects of statins, such as the inhibition of cell proliferation, promotion of apoptosis, and inhibition of inflammation, angiogenesis, and metastasis [16,17,19,20]. The anticancer effects of statins also involve the inhibition of geranylgeranylation, primary of the Rho proteins [16,17]. These findings suggest the possibility of statins playing a role of cancer chemopreventive agents for certain malignancies.

The results of the present study clearly indicated that pitavastatin, which is widely used for the treatment of patients with hyperlipedemia, effectively prevents the development of DEN-induced liver preneoplastic lesions in obese *db/db* mice (Figure 1B). This is the first report that shows the preventive effect of statin analog on the development of obesity-related liver tumorigenesis. The unfavorable effects of obesity and related metabolic abnormalities are serious global healthcare problem. Among them, the promotion of HCC by obesity [1-4] is

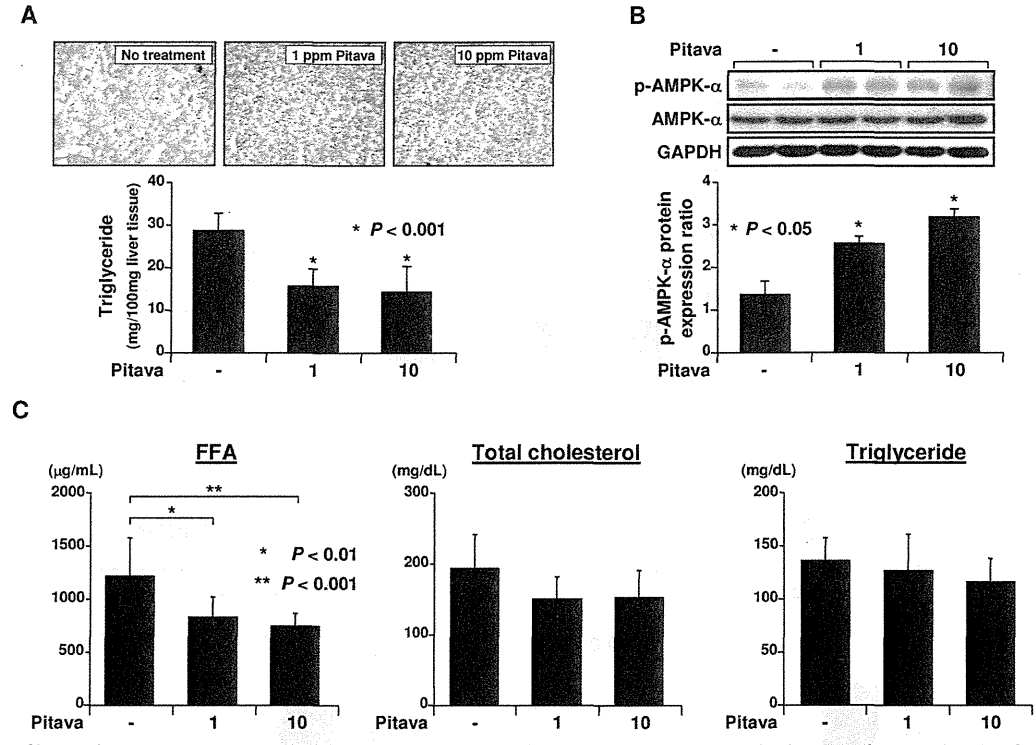

Figure 3 Effects of pitavastatin on hepatic steatosis, activation of the AMPK-α protein in the liver, and serum levels of FFA, total cholesterol, and triglyceride in DEN-treated *db/db* mice. (*A*) Frozen liver sections from experimental mice with or without pitavastatin treatment were stained with Oil red O to show steatosis (upper panels). Hepatic lipids were extracted from the frozen livers of these mice, and the triglyceride levels were measured (lower panel). Values are the means ± SD. * $P < 0.001$ vs. the untreated group. (*B*) The total proteins were extracted from the livers of experimental mice and the expression levels of the AMPK-α and p-AMPK-α proteins were examined by western blot analysis (upper panel). The intensities of the blots were quantified by densitometry (lower panel). GAPDH antibody served as a loading control. Two lanes represent protein samples obtained from 2 different mice in each group. Repeat western blots yielded similar results. Values are the means ± SD. * $P < 0.05$ vs. the untreated group. (*C*) The serum concentrations of FFA, total cholesterol, and triglyceride in all groups. Values are the means ± SD. * $P < 0.01$ and ** $P < 0.001$ vs. the untreated group, respectively.

one of the critical issues that need to be addressed in the management of this malignancy. Therefore, our present finding seems to be clinically significant when considering the prevention of HCC in obese people, who are at an increased risk of developing HCC.

The suppressive effect of pitavastatin on the development of obesity-related liver tumorigenesis was most likely associated with the induction of apoptosis in the liver (Figures. 2A and 2B) and the inhibition of proliferation in FCA (Figure 2C). This inhibition was also associated with the improvement of hepatic steatosis (Figure 3A) and the attenuation of inflammation (Figure 4) because excess accumulation of lipids in the liver accelerates hepatic tumorigenesis by inducing a chronic

Table 2 Serum levels of AST, ALT, adiponectin, and leptin in the experimental mice

Group no.	Treatment	No. of mice	AST[a] (IU/L)	ALT[b] (IU/L)	Adiponectin (μg/mL)	Kidney (ng/dL)
1	DEN alone	12	194 ± 47[c]	291 ± 112	15.5 ± 2.4	108.1 ± 33.4
2	DEN + 1 ppm Pitavastatin	12	111 ± 28[d]	180 ± 49[d]	19.2 ± 4.5[e]	104.3 ± 33.2
3	DEN + 10 ppm Pitavastatin	12	144 ± 28[e]	227 ± 96[e]	21.2 ± 7.4[e]	93.2 ± 31.2

[a]aspartate aminotransferase.

[b]alanine aminotransferase.

[c]Mean ± SD.

[d]Significantly different from Group 1 ($P < 0.001$).

[e]Significantly different from Group 1 ($P < 0.05$).

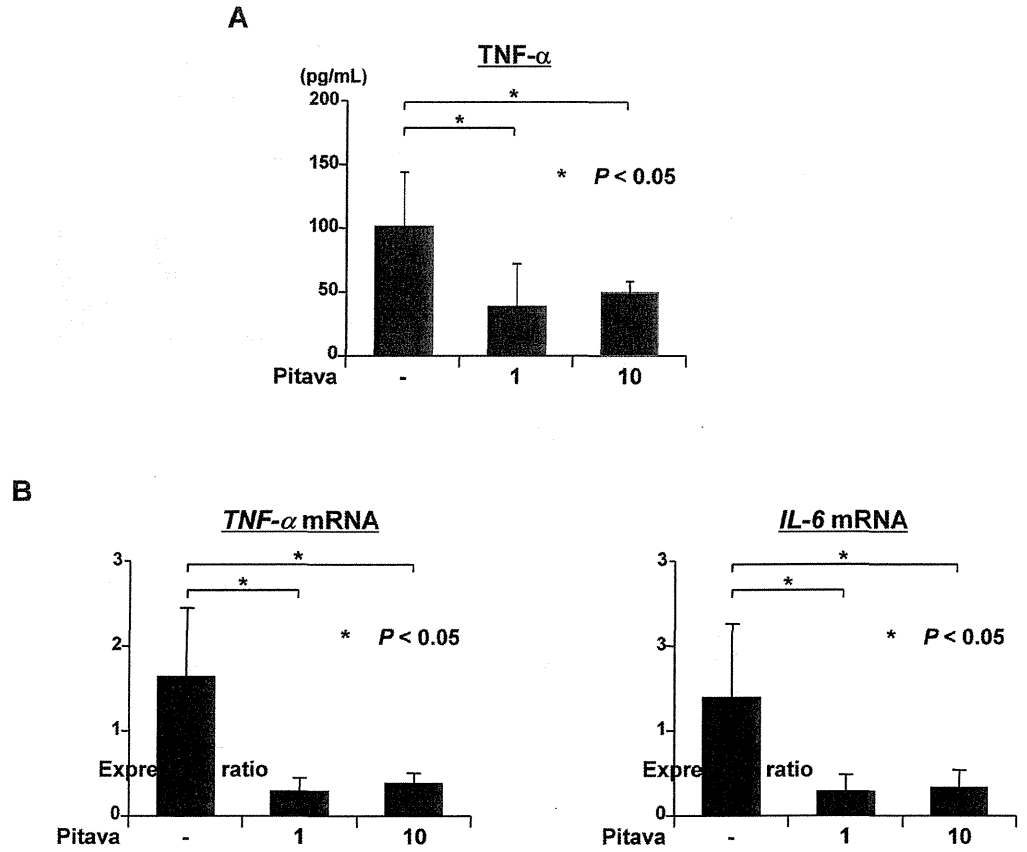

Figure 4 Effects of pitavastatin on the serum levels of TNF-α and the expression levels of *TNF-α* and *IL-6* mRNAs in the liver of DEN-treated *db/db* mice. (*A*) The serum concentration of TNF-α was measured by enzyme immunoassay. Values represent the means ± SD. * $P < 0.05$ vs. the untreated group. (*B*) The total RNAs were isolated from the livers of experimental mice and the expression levels of *TNF-α* and *IL-6* mRNAs were examined by quantitative real-time RT-PCR using specific primers. The expression levels of each mRNA were normalized to the level of *GAPDH* mRNA. Values represent the means ± SD. * $P < 0.05$ vs. the untreated group.

inflammatory reaction [5-7]. Pitavastatin mainly ameliorates hepatic steatosis by decreasing serum FFA levels (Figure 3C) since the high influx of FFA into the liver plays a major role in hepatic fat accumulation [5,6]. In addition, activation of AMPK-α by pitavastatin in the liver (Figure 3B), which increases FA oxidation, decreases FA synthesis, and improves hyperlipidemia [26], also contributes to the inhibition of lipid deposition in the liver. Further, these findings are significant when considering the prevention of obesity-related carcinogenesis because AMPK is regarded as a metabolic tumor suppressor and a promising target for cancer prevention and therapy [27]. AMPK activity is associated with the inhibition of lipogenesis, which has a pathogenic and prognostic significance for HCC [8], induction of apoptosis, and suppression of cell growth in human HCC-derived cells [28]. Pitavastatin has also been shown to inhibit obesity-related colorectal carcinogenesis through the activation of AMPK-α in the colonic mucosa [21].

In the present study, lipid-lowering effects of pitavastatin were positive on serum FFA but not significant on total cholesterol and triglyceride in DEN-treated *db/db* mice (Figure 3C). These findings are consistent with the results of a recent study indicating more high doses of pitavastatin (20 and 40 ppm) did not significantly decrease the serum levels of total cholesterol and triglyceride in Min mice, which show a hyperlipidemic state [29]. On the contrary, Egawa *et al.* [15] demonstrated that pitavastatin administration resulted in a significant reduction in the levels of plasma triglyceride and total cholesterol in aromatase-deficient mice. Treatment with both 1 and 10 ppm pitavastatin for 8 weeks also reduced serum levels of total cholesterol, but not triglyceride, in azoxymethane-treated *db/db* mice [21]. These reports [15,21,29], together with the results of the present study,

suggest that effects of pitavastatin on plasma lipids might depend on the animal strain and experimental procedure. In addition, it has been shown that pitavastatin potently inhibits *de novo* cholesterol synthesis without affecting serum lipid levels [30,31]. In rodents, cholesterol synthesis enzymes were remarkably induced by feedback regulation [32], suggesting that the effects of pitavastatin on reduction of plasma lipid and inhibition of HMG-CoA reductase activity might be masked by such feedback regulation.

Increases in TNF-α and IL-6 levels, which are accompanied by lipid accumulation in the liver, are involved in obesity-related liver carcinogenesis [5-7]. Therefore, reduction of serum TNF-α levels (Figure 4A) and inhibition of the expression of *TNF-α* and *IL-6* mRNAs in the liver (Figure 4B) by pitavastatin are important in preventing obesity-related liver tumorigenesis. These findings are consistent with previous reports that pitavastatin significantly suppresses inflammation- and obesity-related mouse colon carcinogenesis by attenuating chronic inflammation [21,33]. The effects of pitavastatin on decreasing the levels of TNF-α might be largely dependent on the reduction of BMI (Table 1) and serum FFA levels (Figure 3C). These phenomena may also be associated with the improvement of adipocytokine imbalance (Table 2) because TNF-α has been shown to decrease the levels of adiponectin, which is secreted by the adipose tissue, while increasing the levels of leptin in the adipocytes [34,35]. Moreover, up-regulation of serum adiponectin levels (Table 2) also plays a role in attenuating inflammation because this adipocytokine possesses the ability to down-regulate the production of TNF-α and IL-6 [36]. Adiponectin alleviates hepatic steatosis and ALT abnormalities in alcohol-induced fatty liver mice model and in *ob/ob* mice, a NAFLD mice model, by enhancing FA oxidation, while decreasing FA synthesis and TNF-α production in the liver [37]. Hypoadiponectinemia enhances the progression of steatosis and hepatic tumor formation in a mice model of NASH [38]. In addition, adiponectin inhibits cell proliferation and induces apoptosis in human HCC-derived cells by inducing AMPK activation [39]. Therefore, the elevation of adiponectin and activation of AMPK might be effective for the prevention of obesity-related tumorigenesis.

Hepatotoxicity is one of the critical concerns in treatment with statins. In the present study, however, pitavastatin did not cause significant toxicity in the liver as determined by histological examination. The serum aminotransferase (ALT and AST) levels were also decreased by treatment with this agent (Table 2). The safety of statins for patients with liver dysfunction has also been reported in several clinical trials [40]. In addition, patients with chronic liver disease, including NAFLD/NASH and HCV infection, may benefit from statins because cardiovascular risk is likely to be high in these diseases [12,41]. Therefore, statin use might be a promising therapy for NASH patients who have an increased risk of HCC [9], although periodic monitoring of serum aminotransferase levels should be conducted. The result of a recent epidemiological study revealing a significant relationship between the risk reduction of HCC and statin use among diabetic patients [18] may also encourage statin therapy for patients with chronic liver disease, especially NASH patients, who frequently have hyperlipidemia as well as insulin resistance.

Finally, it should be noted that the results of recent studies indicating that supplementation with branched-chain amino acids and acyclic retinoid, both of which exert chemopreventive effects on the development of HCC in clinical trials [3,42], suppresses DEN-induced liver tumorigenesis in *db/db* mice by improving hepatic steatosis and attenuating chronic inflammation [22,43]. In summary, the results of the present study, together with those of the cited reports [22,43], suggest that the prevention of liver carcinogenesis by targeting hepatic steatosis, chronic inflammation, and adipocytokine imbalance, through either pharmaceutical or nutritional intervention, might be a promising strategy for obese individuals who are at an increased risk of developing HCC. Pitavastatin appears to be a potentially effective candidate for this purpose since it can improve liver steatosis and attenuate inflammation, at least in part, through the activation of AMPK-α and up-regulation of adiponectin.

List of abbreviations used

ALT: alanine aminotransferase; AMPK: AMP-activated kinase; ANOVA: analysis of variance; AST: aspartate aminotransferase; BMI: body mass index; DEN: diethylnitrosamine; FA: fatty acid; FCA: foci of cellular alteration; FFA: free fatty acid; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; H&E: hematoxylin & eosin; HCC: hepatocellular carcinoma; HMG-CoA: 3-hydroxy-3-methylglutaryl coenzyme A; IL: interleukin; PCNA: proliferating cell nuclear antigen; RT-PCR: reverse transcription-PCR; TNF-α: tumor necrosis factor-α.

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Authors' contributions

MS, YY, and TT conceived of the study, participated in its design, and drafted the manuscript. MS, YY, HS, MK, DT, AB, and TO performed *in vivo* experiment. TK performed statistical analysis. HT and HM helped to draft the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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