Preventive Effects of (−)-Epigallocatechin Gallate on Diethylnitrosamine-Induced Liver Tumorigenesis in Obese and Diabetic C57BL/KsJ-*db/db* Mice

Masahito Shimizu[1], Hiroyasu Sakai[1], Yohei Shirakami[1], Yoichi Yasuda[1], Masaya Kubota[1], Daishi Terakura[1], Atsushi Baba[1], Tomohiko Ohno[1], Yukihiko Hara[2], Takuji Tanaka[3], and Hisataka Moriwaki[1]

Abstract

Obesity and related metabolic abnormalities, including insulin resistance and a state of chronic inflammation, increase the risk of hepatocellular carcinoma. Abnormal activation of the insulin-like growth factor (IGF)/ IGF-1 receptor (IGF-1R) axis is also involved in obesity-related liver tumorigenesis. In the present study, we examined the effects of (−)-epigallocatechin gallate (EGCG), a major biologically active component of green tea, on the development of diethylnitrosamine (DEN)-induced liver tumorigenesis in C57BL/KsJ-*db/db* (*db/db*) obese mice. Male *db/db* mice were given tap water containing 40 ppm DEN for 2 weeks and then they received drinking water containing 0.1% EGCG for 34 weeks. At sacrifice, drinking water with EGCG significantly inhibited the development of liver cell adenomas in comparison with the control EGCG-untreated group. EGCG inhibited the phosphorylation of the IGF-1R, ERK (extracellular signal-regulated kinase), Akt, GSK-3β (glycogen synthase kinase-3β), Stat3, and JNK (c-Jun NH_2-terminal kinase) proteins in the livers of experimental mice. The serum levels of insulin, IGF-1, IGF-2, free fatty acid, and TNF-α were all decreased by drinking EGCG, which also decreased the expression of TNF-α, interleukin (IL)-6, IL-1β, and IL-18 mRNAs in the livers. In addition, EGCG improved liver steatosis and activated the AMP-activated kinase protein in the liver. These findings suggest that EGCG prevents obesity-related liver tumorigenesis by inhibiting the IGF/IGF-1R axis, improving hyperinsulinemia, and attenuating chronic inflammation. EGCG, therefore, may be useful in the chemoprevention of liver tumorigenesis in obese individuals. *Cancer Prev Res; 4(3); 396–403. ©2011 AACR.*

Introduction

Hepatocellular carcinoma (HCC) is one of the most common and deadly cancers worldwide. Chronic inflammation of the liver and subsequent cirrhosis, which are mainly induced by infection with hepatitis B and hepatitis C viruses, are risk factors for HCC development. Increasing evidence also indicates that obesity and related metabolic abnormalities, especially diabetes mellitus, raise the risk of HCC (1–3). Several pathophysiologic mechanisms linking obesity, steatosis, and liver carcinogenesis have been shown, including the emergence of insulin resistance and the subsequent inflammatory cascade. Insulin resistance leads to an increased expression of TNF-α, a central

mediator of chronic inflammatory diseases, and its dysregulation is associated with the development of steatosis and inflammation within the liver (4, 5). Hyperinsulinemia also upregulates the levels of insulin-like growth factors (IGF) and abnormal activation of the IGF/IGF-1 receptor (IGF-1R) axis contributes to the development of various types of human malignancies, including HCC (6, 7). These findings suggest that targeting insulin resistance may be an effective strategy for preventing the development of obesity-related HCC. A recent animal experiment revealed that supplementation with branched chain amino acids, which is used to improve protein malnutrition in patients with liver cirrhosis, prevents obesity-related liver tumorigenesis by targeting insulin resistance and the IGF/IGF-1R axis (8).

Green tea, a beverage commonly consumed worldwide, possesses anticancer and cancer chemopreventive properties, and (−)-epigallocatechin gallate (EGCG) is the most potent of the green tea catechins (GTC) with respect to exerting these beneficial effects (9, 10). EGCG inhibits cell proliferation and induces apoptosis in cancer cells by inhibiting activation of some types of receptor tyrosine kinases (RTK) and related downstream signaling pathways (11, 12). Among such RTKs, the IGF-1R is one of the critical targets of EGCG with respect to its anticancer effects. In

Authors' Affiliations: [1]Department of Medicine, Gifu University Graduate School of Medicine, Gifu; [2]Tea Solution, Hara Office Inc., Tokyo; and [3]Department of Oncologic Pathology, Kanazawa Medical University, Ishikawa, Japan

Corresponding Author: Masahito Shimizu, Department of Medicine, Gifu University Graduate School of Medicine, 1-1 Yanagido, Gifu 501-1194, Japan. Phone: 81-(58)-230-6313; Fax: 81-(58)-230-6310; E-mail: shimim-gif@umin.ac.jp

American Association for **Cancer Research**

human HCC- and colon cancer–derived cells, EGCG suppresses cell growth by inhibiting the activation of the IGF/IGF-1R axis and its downstream ERK (extracellular signal-regulated kinase) and Akt proteins (13–15). EGCG also overcomes the activation of the IGF/IGF-1R axis and thereby inhibits the development of colonic premalignant lesions in an obesity-related colon carcinogenesis model (16).

In addition to anticancer and cancer chemopreventive effects, GTCs, especially EGCG, seem to have antiobesity and antidiabetic effects (17, 18). GTCs also possess anti-inflammatory properties because they inhibit the expression of proinflammatory cytokines TNF-α and interleukin (IL)-6, which are also associated with cancer prevention by GTCs (19–21). Supplementation with GTCs decreases plasma levels of insulin, TNF-α, and IL-6 in a high-fructose diet-induced rat insulin resistance model (22). These reports suggest the possibility that long-term treatment with GTCs may be effective for preventing the progression of obesity-related diseases, including the development of HCC. In the present study, we examined the effects of EGCG on obesity-related liver tumorigenesis in male C57BL/KsJ-db/db (db/db) mice initiated with diethylnitrosamine (DEN) by focusing on the inhibition of the activation of the IGF/IGF-1R axis. We also investigated whether EGCG treatment improves hyperinsulinemia, liver steatosis, and inflammatory condition in this preclinical mouse model that can be used to evaluate the mechanisms underlying the inhibition of obesity-related liver tumorigenesis by candidate chemopreventive agents (8).

Materials and Methods

Animals and chemicals

Four-week-old male db/db mice were obtained from Japan SLC, Inc., and were humanely maintained at Gifu University Life Science Research Center in accordance with the Institutional Animal Care Guidelines. DEN was purchased from Sigma Chemical Co. EGCG was obtained from Mitsui Norin Co. Ltd.

Experimental procedure

At 5 weeks of age, a total of 30 db/db mice were randomly divided into the following 4 experimental and control groups: DEN alone (group 1, $n = 10$); DEN plus 0.1% EGCG (group 2, $n = 10$); 0.1% EGCG alone (group 3, $n = 5$); and no treatment (group 4, $n = 5$). All of the mice in groups 1 and 2 were given tap water containing 40 ppm DEN for the first 2 weeks of the experiment, which is sufficient to develop hepatocellular neoplasms in db/db mice (8). After DEN treatment, the mice in group 2 were given free access to tap water containing 0.1% EGCG until the end of the experiment. The mice in group 3 were given 0.1% EGCG throughout the experiment. The concentration of EGCG (0.1%), which was established according to the findings of previous chemopreventive studies (16, 23), was within the physiologic range after daily intake of GTCs in human per unit body weight basis (24). The mice in groups

1 and 4 were given tap water without EGCG. At 41 weeks of age (after 34 weeks of EGCG treatment), all of the mice were sacrificed to analyze the development of liver neoplasms and preneoplastic lesions.

Histopathologic analysis

At sacrifice, the livers were immediately removed and macroscopically inspected for the presence of neoplasms. Maximum sagittal sections of each lobe (6 sublobes) were used for histopathologic examination. For all experimental groups, 4-μm thick sections, prepared from formalin-fixed and paraffin-embedded tissue blocks, were subjected to hematoxylin and eosin staining for histopathology. The presence of HCC, liver cell adenoma, and foci of cellular alterations (FCA) was judged according to previously described criteria (25). The multiplicity of FCA was assessed on a per unit area (cm^2) basis.

Protein extraction and Western blot analysis

Total protein was extracted from the nontumorous areas of livers and equivalent amounts of proteins (20 μg/lane) were examined by a Western blot analysis (8). Previously described primary antibodies for IGF-1R, phosphorylated IGF-1R (p-IGF-1R), ERK, p-ERK, Akt, p-Akt, Stat3, p-Stat3, AMP-activated kinase (AMPK), p-AMPK, glycogen synthase kinase (GSK)-3β, p-GSK-3β, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used (16, 26, 27). The primary antibody for c-Jun NH_2-terminal kinase (JNK) and p-JNK was obtained from Cell Signaling Technology. GAPDH served as a loading control.

RNA extraction and quantitative real-time reverse transcriptase PCR

Total RNA was isolated from the nontumorous areas of livers by using the RNAqueous-4PCR kit (Ambion Applied Biosystems). The cDNA was amplified from 0.2 μg of total RNA, using the SuperScript III First-Strand Synthesis System (Invitrogen). Quantitative real-time reverse transcriptase PCR (RT-PCR) analysis was done using specific primers that amplify the TNF-α, IL-6, IL-1β, IL-18, and β-actin genes, as described previously (26, 28).

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 concentrations of insulin (Shibayagi), TNF-α, (Shibayagi), IGF-1 (R&D Systems), and IGF-2 (R&D Systems) were determined by an enzyme immunoassay according to the manufacturers' protocols. The serum levels of free fatty acid (FFA) were measured with a standard clinical automatic analyzer (type 7180; Hitachi).

Hepatic lipid analysis

Approximately 200 mg of frozen liver was homogenized, and lipids were extracted using Folch's method (29). The triglyceride levels in the liver were measured using the triglyceride E-test kit (Wako Pure Chemical Co.) according to the manufacturers' protocol. To visualize the intrahepatic

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

Group no.	Treatment	No. of mice	Body wt, g	Relative wt, g/100g body wt	
				Liver	Fat[a]
1	DEN alone	10	73.3 ± 8.8[b]	6.1 ± 1.6	10.6 ± 2.1
2	DEN + 0.1% EGCG	10	71.6 ± 8.1	6.1 ± 1.3	7.4 ± 1.5[c]
3	0.1% EGCG alone	5	61.1 ± 7.1	7.3 ± 1.5	9.3 ± 1.2
4	Tap water	5	67.9 ± 7.9	7.1 ± 1.5	9.0 ± 1.4

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

lipids, Sudan III staining was carried out using the standard procedure with frozen liver sections.

Statistical analysis

The results are presented as the means ± 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 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

During the experiment, EGCG treatment in drinking water did not cause any clinical symptoms for toxicity. No significant differences were observed in the body weights or relative weights of the livers among the 4 groups at the end of the study (Table 1). In the DEN-treated groups, drinking EGCG (group 2) significantly reduced

the relative weights of white adipose tissue (periorchis and retroperitoneum) as compared with the untreated group (group 1, $P < 0.01$) at the termination of the experiment. Histopathologic examination revealed the absence of toxicity of EGCG in the liver, kidney, and spleen (data not shown).

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

The incidence and multiplicity of liver neoplasms (adenoma and HCC) and FCA in the mice of all groups are summarized in Table 2. Irrespective of DEN treatment, FCA developed in the livers of mice from all groups. However, the number of this preneoplastic lesion was significantly increased by treatment with DEN ($P < 0.001$). In the DEN-treated mice, EGCG in drinking water significantly inhibited the development of FCA in comparison with the untreated control mice ($P < 0.001$). The incidence ($P < 0.01$) and multiplicity ($P < 0.01$) of adenoma, which developed only in the DEN-treated mice, were also significantly decreased by EGCG. HCC developed only in the DEN-treated groups, but the incidence (10% in each group) was not high. These findings might suggest that the duration of the experiments (41 weeks) was sufficient

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

| Group no.	Treatment	No. of mice	Incidence		Multiplicity[a]		FCA, no./cm²
			Adenoma	HCC	Adenoma	HCC	
1	DEN alone	10	7/10 (70%)	1/10 (10%)	1.4 ± 1.2[b]	0.1 ± 0.3	14.9 ± 4.2[c]
2	DEN + 0.1% EGCG	10	1/10 (10%)[d]	1/10 (10%)	0.1 ± 0.3[e]	0.1 ± 0.3	7.7 ± 3.0[f]
3	0.1% EGCG alone	5	0/5 (0%)	0/5 (0%)	0	0	5.8 ± 1.3
4	Tap water	5	0/5 (0%)	0/5 (0%)	0	0	8.2 ± 1.1

[a]Number of neoplasms per mouse.
[b]Mean ± SD.
[c]Significantly different from group 4 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 the Tukey–Kramer multiple comparison test ($P < 0.01$).
[f]Significantly different from group 1 by the Tukey–Kramer multiple comparison test ($P < 0.001$).
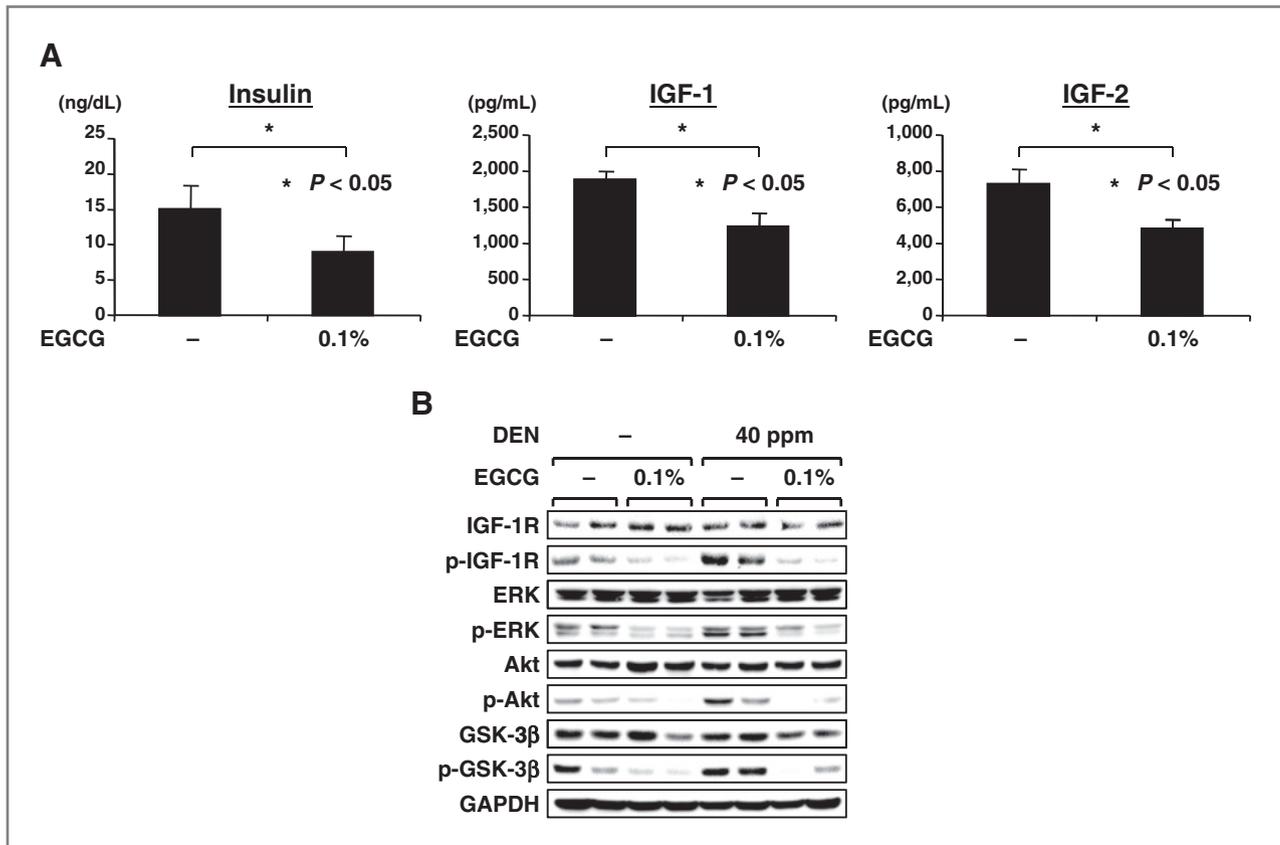
Figure 1. Effects of EGCG on the levels of serum insulin, IGF-1, and IGF-2 and on the activation of the IGF/IGF-1R axis in the liver of experimental mice. A, the serum concentrations of insulin, IGF-1, and IGF-2 in DEN-treated *db/db* mice were measured by an enzyme immunoassay. Values are the means ± SD. *, $P < 0.05$ versus the untreated group. B, the total proteins were extracted from the livers of experimental mice and the expression levels of the IGF-1R, p-IGF-1R, ERK, p-ERK, Akt, p-Akt, GSK-3β, and p-GSK-3β proteins were examined by a Western blot analysis, using the respective antibodies. Equal protein loading was verified by the detection of GAPDH. Two lanes represent protein samples from 2 different mice from each group. Repeat Western blots yielded similar results.

to develop adenoma but was relatively short to induce substantial number of HCC in the present study.

Effects of EGCG on the serum levels of insulin, IGF-1, and IGF-2 and on the phosphorylation of IGF-1R, ERK, Akt, and GSK-3β proteins in the livers of experimental mice

Hyperinsulinemia and abnormal activation of the IGF/IGF-1R axis play a critical role in obesity-related liver carcinogenesis (6, 7). Therefore, the effects of EGCG on the serum levels of insulin, IGF-1, and IGF-2 and the activation of IGF-1R protein in the liver of experimental mice were examined. As shown in Figure 1A, the administration of EGCG in the drinking water significantly decreased the serum levels of insulin, IGF-1, and IGF-2 ($P < 0.05$, respectively) in DEN-treated mice. Western blot analysis revealed that IGF-1R protein was phosphorylated (i.e., activated) by the administration of DEN but EGCG drinking decreased the levels of p-IGF-1R protein in the livers of experimental mice irrespective of DEN treatment. The levels of the phospholylated forms of the ERK and Akt proteins, which are located downstream of IGF-1R (30),

were also decreased by EGCG drinking. In addition, the phospholylation of GSK-3β, which is mediated by the IGF-1R/Akt signaling pathway (31), was significantly inhibited by EGCG drinking. DEN treatment increased the levels of p-ERK, p-Akt, and p-GSK-3β proteins, but the inhibitory effects of EGCG on the expression of these proteins were not affected by the administration of this carcinogen (Fig. 1B). These findings indicate that DEN enhances liver tumorigenesis in *db/db* mice, at least in part, by activating the IGF/IGF-1R axis and EGCG may inhibit the development of obesity-related liver neoplasms by targeting hyperinslinemia and the activation of the IGF/IGF-1R axis.

Effects of EGCG on the serum levels of FFA, hepatic steatosis, and the activation of AMPK protein in the livers of DEN-treated *db/db* mice

Hepatic steatosis, which is caused by hyperinsulinemia and an increased FFA concentration, is considered to be involved in liver tumorigenesis (4, 5). Therefore, the effects of EGCG on the serum levels of FFA and accumulation of lipids in the liver of DEN-treated *db/db* mice were examined. The levels of FFA in serum were significantly
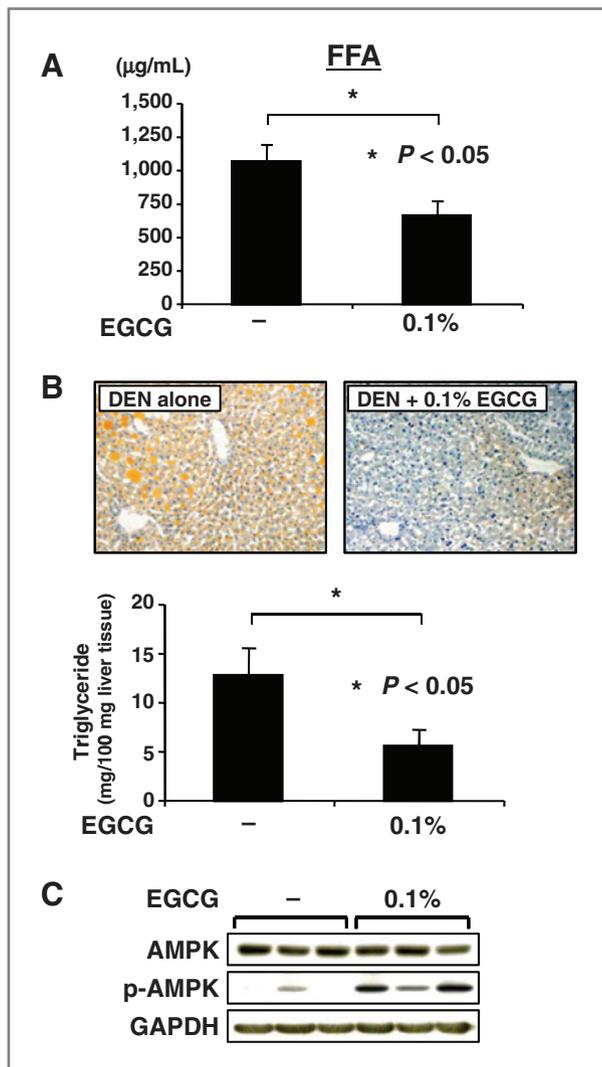
Figure 2. Effects of EGCG on the serum levels of FFA, hepatic steatosis, and the activation of the AMPK protein in the liver of DEN-treated *db/db* mice. A, the serum concentration of FFA was measured by an enzymatic method. Values are the means ± SD. *, *P* < 0.05 versus the untreated group. B, frozen liver sections from DEN-exposed mice with or without EGCG treatment 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). Values are the means ± SD. *, *P* < 0.05 versus the untreated group. C, 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 a Western blot analysis. GAPDH antibody served as a loading control. Three lanes represent protein samples from 3 different mice from the untreated and 0.1% EGCG-treated groups, respectively.

decreased by EGCG drinking (Fig. 2A, *P* < 0.05). The examination of Sudan III–stained sections showed that EGCG markedly improved the accumulation of lipids in the livers of DEN-treated mice (Fig. 2B, top panels). Similar to the histologic findings, the levels of triglyceride in the liver were significantly decreased by the administration of EGCG (Fig. 2B, bottom panel, *P* < 0.05). In addition, the expression levels of p-AMPK proteins were significantly

increased by EGCG, thus indicating that the agent activated the AMPK protein, a central signaling system controlling the pathways of lipid metabolism (32), in the livers of the experimental mice (Fig. 2C).
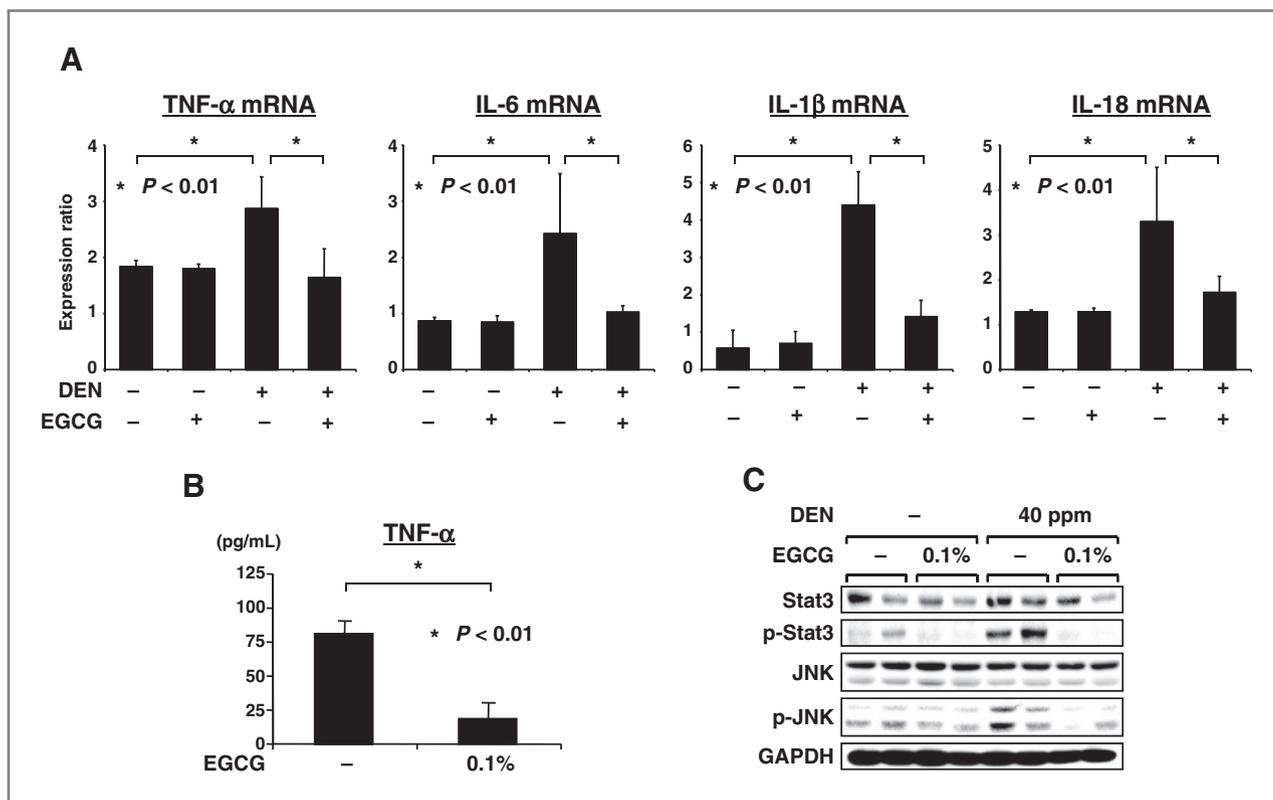
Effects of EGCG on the hepatic expression of TNF-α, IL-6, IL-1β, and IL-18 mRNAs, serum levels of TNF-α, and the phosphorylation of Stat3 and JNK proteins in the livers of experimental mice

Obesity promotes liver tumorigenesis by inducing inflammation (33). Therefore, whether drinking EGCG altered the levels of the inflammatory mediators in the experimental mice was examined. As shown in Figure 3A, quantitative real-time RT-PCR revealed that the expression levels of TNF-α, IL-6, IL-1β, and IL-18 mRNAs in the livers, which were increased by DEN treatment ($P \leq 0.01$, respectively), were significantly decreased by EGCG ($P \leq 0.01$, respectively). The serum levels of TNF-α were also reduced after EGCG drinking in DEN-treated mice (Fig. 3B, $P <$ 0.01). Furthermore, irrespective of DEN treatment, EGCG drinking decreased the expression levels of the p-Stat3 and p-JNK proteins, which play a role in obesity/TNF-α–mediated hepatic inflammation (34, 35) and are increased by DEN, in the livers of experimental mice (Fig. 3C). These findings suggest that EGCG improves hepatic steatosis and attenuates liver inflammation, which might be enhanced by DEN, in obese and diabetic *db/db* mice.

Discussion

Obesity and related metabolic abnormalities, particularly diabetes mellitus and insulin resistance, are significant risk factors for the development of HCC and therefore may be promising targets for the prevention of this malignancy in obese individuals (1–3, 8). The results of the present study clearly indicated that EGCG, which has been shown to improve dysregulation of energy homeostasis (17, 18), effectively prevents the development of liver tumorigenesis in obese and diabetic *db/db* mice by improving hyperinsulinemia and hepatic steatosis. A recent study showed that EGCG suppressed the development of colonic premalignant lesions induced by azoxymethane in *db/db* mice through improvement of hyperinsulinemia and inhibition of the IGF/IGF-1R axis on the colonic mucosa (16). These findings suggest that the improvement of metabolic abnormalities by either pharmaceutical or nutritional intervention may be an effective strategy to prevent certain types of obesity-related carcinogenesis and EGCG is a promising candidate for this purpose.

We showed that several biological activities of EGCG might contribute to the inhibition of obesity-related liver tumorigenesis in the present study. Among them, it should be emphasized first that EGCG decreases the serum levels of insulin, IGF-1, and IGF-2 while also inhibiting the activation of IGF-1R and related downstream signaling pathways, including the MAPK (mitogen-activated protein kinase)/ERK and PI3K (phosphatidylinositol 3-kinase)/Akt

Figure 3. Effects of EGCG on the expression levels of TNF-α, IL-6, IL-18, and IL-1β mRNAs, the serum levels of TNF-α, and the activation of Stat3 and JNK proteins in the liver of experimental mice. A, the total RNAs were isolated from the livers of experimental mice, and the expression levels of TNF-α, IL-6, IL-1β, and IL-18 mRNAs 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 means ± SD. *, $P < 0.01$ versus the control groups. B, the serum concentration of TNF-α in DEN-treated db/db mice was measured by enzyme immunoassay. Values are the means ± SD. *, $P < 0.01$ versus the untreated group. C, the total proteins were extracted from the livers of experimental mice and the expression levels of the Stat3, p-Stat3, JNK, and p-JNK proteins were examined by a Western blot analysis. GAPDH antibody served as a loading control.

pathways, in the livers of experimental mice. These findings seem to be significant because the alteration of the IGF/IGF-1R axis, which is induced by insulin resistance, is involved in liver carcinogenesis and thus might play a critical role as a molecular target for HCC chemoprevention (6–8). In human HCC–derived cells, IGF-1 and IGF-2 activate IGF-1R, ERK, and Akt proteins and increase the expression of IGF-1 and IGF-2 mRNAs themselves but EGCG inhibits these sequences and thus suppresses growth and induces apoptosis in HCC cells (13). These findings, together with the results of the present study, suggest the possibility that EGCG overcomes the stimulatory effects of IGFs, disrupts the IGF/IGF-1R–related autocrine/paracrine loops, and thereby prevents the development of obesity-related liver tumorigenesis. In addition, the inhibition of GSK-3β phosphorylation by EGCG also plays a role in preventing the development of liver neoplasms because phosphorylation of this kinase, which is mediated by the IGF-1R/Akt axis, is closely associated with liver carcinogenesis (31).

Excess accumulation of lipids in the liver accelerates HCC development (4, 5). Therefore, the improvement of hepatic steatosis by EGCG is also significant when considering the inhibitory effects of this agent on obesity-related liver tumorigenesis. This effect of EGCG may be associated with reductions in white adipose tissue and serum FFA levels because host factors, particularly increased visceral fat and a high influx of FFA to the liver, lead to hepatic fat accumulation (4, 5). In addition, EGCG may also improve metabolic abnormalities by activating AMPK in the liver, which enhances insulin sensitivity and increases fatty acid oxidation but decreases fatty acid synthesis (32). This finding is consistent with recent studies showing that EGCG increases insulin sensitivity and fat oxidation and induces AMPK activity in the liver (36, 37). Furthermore, in addition to the improvement of metabolic disorders, activation of AMPK by EGCG also positively contributes to the prevention of hepatotumorigenesis because decreased AMPK activation is implicated in tumor development and therefore may be a tumor suppressor and a promising target for cancer chemoprevention (38). In fact, EGCG has been shown to inhibit lipogenesis and cell-cycle progression through the activation of AMPK in human HCC–derived cells (39). The phosphorylation of LKB1, which is a tumor suppressor protein and a major AMPK kinase (38), is also increased by EGCG (37). Thus,

the antiobesity and cancer chemopreventive effects of EGCG might be mediated, at least in part, by the activation of AMPK.

Insulin resistance and lipid accumulation in the liver, which is mainly induced by the FFA flux, promotes liver inflammation through the production of proinflammatory cytokines such as TNF-α and IL-6, and this chronic inflammatory response is closely associated with activation of Stat3 and increased risk of HCC (4, 5, 33). Therefore, decreases in the expression of TNF-α, IL-6, IL-1β, and IL-18 mRNAs in the liver, reduced levels of serum TNF-α, and inhibited activation of Stat3 in the liver of *db/db* mice treated with EGCG are considered to be important in preventing obesity-related liver tumorigenesis. Among these targets, TNF-α, which links obesity with insulin resistance and contributes to obesity-induced IL-6 production (33, 34), has been shown to be a crucial target of EGCG that can inhibit cancer cell growth and prevent inflammation-related colorectal carcinogenesis (19–21). The inhibition of the activation of the IL-6/Stat3 axis by EGCG is also important because this axis plays a critical role in HCC development (40, 41). In addition, the effect of EGCG to inhibit JNK activation, which is caused by higher levels of TNF-α and FFA and is involved in obesity-mediated insulin resistance (42), also contributes to the prevention of obesity-related liver tumorigenesis by EGCG because JNK seems to be one of the most important kinases that is upregulated in HCC and could thus be a potential therapeutic target for this malignancy (43). Because JNK is located downstream of IGF-IR (30), the inhibition of the activation of the IGF/IGF-1R axis may also lead to the indirect inhibition of JNK.

One of the effective strategies for HCC chemoprevention is the deletion of latent malignant clones before they progress to detectable neoplasms, and improvement of whole liver condition might play a role in this prevention (44, 45). The liver accumulated with fat, which activates the IGF/IGF-1R axis and induces chronic inflammation, might be regarded as a hypercarcinogenic field (4, 5, 8, 33). Therefore, the findings that EGCG inhibits the activation of IGF-1R and related downstream signaling pathways and ameliorates inflammatory condition in nontumorous hepatic tissues seem to be significant when considering the practice of HCC chemoprevention. Presumably, EGCG reduces the number of FCA, at least in part, by improving the condition in the whole liver and thus preventing obesity-related field tumorigenesis of the liver in the present study.

The beneficial effects of GTCs have been reported in several clinical trials. For instance, supplementation with GTCs can significantly prevent the development of both colorectal adenomas and prostate cancers without causing adverse effects (46, 47). A double-blind, placebo-controlled pilot study showed that EGCG has the potential to increase fat oxidation in men (48), although more studies with a larger sample size are required to confirm this effect. The results of these trials may encourage the clinical usage of GTCs for obese patients to prevent pathogenesis of various chronic diseases that are caused by excessive body weights. In summary, the prevention of HCC by targeting the IGF/IGF-1R axis, hepatic steatosis, and chronic inflammation, which are caused by dysregulation of energy homeostasis, might represent a promising strategy for obese individuals who are at an increased risk of developing HCC (3, 8). GTCs, including EGCG, seem to be potentially effective and critical candidates for this purpose because, as shown in the results of the present study and those from previous reports, these agents can target metabolic abnormalities and may therefore restore metabolic homeostasis (16–22).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Grant Support

References

1. El-Serag HB, Rudolph KL. Hepatocellular carcinoma: epidemiology and molecular carcinogenesis. Gastroenterology 2007;132:2557–76.
2. El-Serag HB, Tran T, Everhart JE. Diabetes increases the risk of chronic liver disease and hepatocellular carcinoma. Gastroenterology 2004;126:460–8.
3. Muto Y, Sato S, Watanabe A, Moriwaki H, Suzuki K, Kato A, et al. Overweight and obesity increase the risk for liver cancer in patients with liver cirrhosis and long-term oral supplementation with branched-chain amino acid granules inhibits liver carcinogenesis in heavier patients with liver cirrhosis. Hepatol Res 2006;35:204–14.
4. Powell EE, Jonsson JR, Clouston AD. Steatosis: co-factor in other liver diseases. Hepatology 2005;42:5–13.
5. Siegel AB, Zhu AX. Metabolic syndrome and hepatocellular carcinoma: two growing epidemics with a potential link. Cancer 2009;115:5651–61.
6. Calle EE, Kaaks R. Overweight, obesity and cancer: epidemiological evidence and proposed mechanisms. Nat Rev Cancer 2004;4:579–91.
7. Alexia C, Fallot G, Lasfer M, Schweizer-Groyer G, Groyer A. An evaluation of the role of insulin-like growth factors (IGF) and of type-I IGF receptor signalling in hepatocarcinogenesis and in the resistance of hepatocarcinoma cells against drug-induced apoptosis. Biochem Pharmacol 2004;68:1003–15.
8. Iwasa J, Shimizu M, Shiraki M, Shirakami Y, Sakai H, Terakura Y, et al. Dietary supplementation with branched-chain amino acids

suppresses diethylnitrosamine-induced liver tumorigenesis in obese and diabetic C57BL/KsJ-db/db mice. Cancer Sci 2010;101:460–7.

9. Yang CS, Wang X, Lu G, Picinich SC. Cancer prevention by tea: animal studies, molecular mechanisms and human relevance. Nat Rev Cancer 2009;9:429–39.

10. Yang CS, Maliakal P, Meng X. Inhibition of carcinogenesis by tea. Annu Rev Pharmacol Toxicol 2002;42:25–54.

11. Shimizu M, Weinstein IB. Modulation of signal transduction by tea catechins and related phytochemicals. Mutat Res 2005;591:147–60.

12. Khan N, Afaq F, Saleem M, Ahmad N, Mukhtar H. Targeting multiple signaling pathways by green tea polyphenol (−)-epigallocatechin-3-gallate. Cancer Res 2006;66:2500–5.

13. Shimizu M, Shirakami Y, Sakai H, Tatebe H, Nakagawa T, Hara Y, et al. EGCG inhibits activation of the insulin-like growth factor (IGF)/IGF-1 receptor axis in human hepatocellular carcinoma cells. Cancer Lett 2008;262:10–8.

14. Shimizu M, Deguchi A, Hara Y, Moriwaki H, Weinstein IB. EGCG inhibits activation of the insulin-like growth factor-1 receptor in human colon cancer cells. Biochem Biophys Res Commun 2005;334:947–53.

15. Shimizu M, Shirakami Y, Sakai H, Yasuda Y, Kubota M, Adachi S, et al. (−)-Epigallocatechin gallate inhibits growth and activation of the VEGF/VEGFR axis in human colorectal cancer cells. Chem Biol Interact 2010;185:247–52.

16. Shimizu M, Shirakami Y, Sakai H, Adachi S, Hata K, Hirose Y, et al. (−)-Epigallocatechin gallate suppresses azoxymethane-induced colonic premalignant lesions in male C57BL/KsJ-db/db mice. Cancer Prev Res 2008;1:298–304.

17. Kao YH, Chang HH, Lee MJ, Chen CL. Tea, obesity, and diabetes. Mol Nutr Food Res 2006;50:188–210.

18. Wolfram S, Raederstorff D, Preller M, Wang Y, Teixeira SR, Riegger C, et al. Epigallocatechin gallate supplementation alleviates diabetes in rodents. J Nutr 2006;136:2512–8.

19. Suganuma M, Sueoka E, Sueoka N, Okabe S, Fujiki H. Mechanisms of cancer prevention by tea polyphenols based on inhibition of TNF-alpha expression. Biofactors 2000;13:67–72.

20. Sueoka N, Suganuma M, Sueoka E, Okabe S, Matsuyama S, Imai K, et al. A new function of green tea: prevention of lifestyle-related diseases. Ann N Y Acad Sci 2001;928:274–80.

21. Shirakami Y, Shimizu M, Tsurumi H, Hara Y, Tanaka T, Moriwaki H. EGCG and polyphenon E attenuate inflammation-related mouse colon carcinogenesis induced by AOM plus DDS. Mol Med Rep 2008;1:355–61.

22. Qin B, Polansky MM, Harry D, Anderson RA. Green tea polyphenols improve cardiac muscle mRNA and protein levels of signal pathways related to insulin and lipid metabolism and inflammation in insulin-resistant rats. Mol Nutr Food Res 2010;54 Suppl 1:S14–23.

23. Shirakami Y, Shimizu M, Adachi S, Sakai H, Nakagawa T, Yasuda Y, et al. (−)-Epigallocatechin gallate suppresses the growth of human hepatocellular carcinoma cells by inhibiting activation of the vascular endothelial growth factor-vascular endothelial growth factor receptor axis. Cancer Sci 2009;100:1957–62.

24. Wang ZY, Agarwal R, Bickers DR, Mukhtar H. Protection against ultraviolet B radiation-induced photocarcinogenesis in hairless mice by green tea polyphenols. Carcinogenesis 1991;12:1527–30.

25. Frith CH, Ward JM, Turusov VS. Tumours of the liver. In: Turusor VS, Mohr U, editors. Pathology of Tumors in Laboratory Animals. Vol 2. Lyon, France: IARC Scientific Publications; 1994. p. 223–70.

26. Yasuda Y, Shimizu M, Shirakami Y, Sakai H, Kubota M, Hata K, et al. Pitavastatin inhibits azoxymethane-induced colonic preneoplastic lesions in C57BL/KsJ-db/db obese mice. Cancer Sci 2010;101:1701–7.

27. Tatebe H, Shimizu M, Shirakami Y, Tsurumi H, Moriwaki H. Synergistic growth inhibition by 9-cis-retinoic acid plus trastuzumab in human hepatocellular carcinoma cells. Clin Cancer Res 2008;14:2806–12.

28. Sakai H, Yamada Y, Shimizu M, Saito K, Moriwaki H, Hara A. Genetic ablation of TNFalpha demonstrates no detectable suppressive effect on inflammation-related mouse colon tumorigenesis. Chem Biol Interact 2010;184:423–30.

29. Folch J, Lees M, Sloane Stanley GH. A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem 1957;226:497–509.

30. Lin Y, Yang Q, Wang X, Liu ZG. The essential role of the death domain kinase receptor-interacting protein in insulin growth factor-I-induced c-Jun N-terminal kinase activation. J Biol Chem 2006;281:23525–32.

31. Desbois-Mouthon C, Blivet-Van Eggelpoël MJ, Beurel E, Boissan M, Delélo R, Cadoret A, et al. Dysregulation of glycogen synthase kinase-3beta signaling in hepatocellular carcinoma cells. Hepatology 2002;36:1528–36.

32. Hardie DG. AMP-activated/SNF1 protein kinases: conserved guardians of cellular energy. Nat Rev Mol Cell Biol 2007;8:774–85.

33. Park EJ, Lee JH, Yu GY, He G, Ali SR, Holzer RG, et al. Dietary and genetic obesity promote liver inflammation and tumorigenesis by enhancing IL-6 and TNF expression. Cell 2010;140:197–208.

34. Hotamisligil GS, Peraldi P, Budavari A, Ellis R, White MF, Spiegelman BM. IRS-1-mediated inhibition of insulin receptor tyrosine kinase activity in TNF-alpha- and obesity-induced insulin resistance. Science 1996;271:665–8.

35. Tuncman G, Hirosumi J, Solinas G, Chang L, Karin M, Hotamisligil GS. Functional in vivo interactions between JNK1 and JNK2 isoforms in obesity and insulin resistance. Proc Natl Acad Sci USA 2006;103:10741–6.

36. Lin CL, Lin JK. Epigallocatechin gallate (EGCG) attenuates high glucose-induced insulin signaling blockade in human hepG2 hepatoma cells. Mol Nutr Food Res 2008;52:930–9.

37. Murase T, Misawa K, Haramizu S, Hase T. Catechin-induced activation of the LKB1/AMP-activated protein kinase pathway. Biochem Pharmacol 2009;78:78–84.

38. Shackelford DB, Shaw RJ. The LKB1-AMPK pathway: metabolism and growth control in tumour suppression. Nat Rev Cancer 2009;9:563–75.

39. Huang CH, Tsai SJ, Wang YJ, Pan MH, Kao JY, Way TD. EGCG inhibits protein synthesis, lipogenesis, and cell cycle progression through activation of AMPK in p53 positive and negative human hepatoma cells. Mol Nutr Food Res 2009;53:1156–65.

40. Naugler WE, Sakurai T, Kim S, Maeda S, Kim K, Elsharkawy AM, et al. Gender disparity in liver cancer due to sex differences in MyD88-dependent IL-6 production. Science 2007;317:121–4.

41. He G, Yu GY, Temkin V, Ogata H, Kuntzen C, Sakurai T, et al. Hepatocyte IKKbeta/NF-kappaB inhibits tumor promotion and progression by preventing oxidative stress-driven STAT3 activation. Cancer Cell 2010;17:286–97.

42. Hirosumi J, Tuncman G, Chang L, Görgün CZ, Uysal KT, Maeda K, et al. A central role for JNK in obesity and insulin resistance. Nature 2002;420:333–6.

43. Chen F, Beezhold K, Castranova V. JNK1, a potential therapeutic target for hepatocellular carcinoma. Biochim Biophys Acta 2009;1796:242–51.

44. Shimizu M, Takai K, Moriwaki H. Strategy and mechanism for the prevention of hepatocellular carcinoma: phosphorylated retinoid X receptor alpha is a critical target for hepatocellular carcinoma chemoprevention. Cancer Sci 2009;100:369–74.

45. Shimizu M, Sakai H, Shirakami Y, Iwasa J, Yasuda Y, Kubota M, et al. Acyclic retinoid inhibits diethylnitrosamine-induced liver tumorigenesis in obese and diabetic C57BL/KsJ-db/db mice. Cancer Prev Res 2011;4:128–36.

46. Shimizu M, Fukutomi Y, Ninomiya M, Nagura K, Kato T, Araki H, et al. Green tea extracts for the prevention of metachronous colorectal adenomas: a pilot study. Cancer Epidemiol Biomarkers Prev 2008;17:3020–5.

47. Bettuzzi S, Brausi M, Rizzi F, Castagnetti G, Peracchia G, Corti A. Chemoprevention of human prostate cancer by oral administration of green tea catechins in volunteers with high-grade prostate intraepithelial neoplasia: a preliminary report from a one-year proof-of-principle study. Cancer Res 2006;66:1234–40.

48. Boschmann M, Thielecke F. The effects of epigallocatechin-3-gallate on thermogenesis and fat oxidation in obese men: a pilot study. J Am Coll Nutr 2007;26:389S–95S.

Preventive Effects of (−)-Epigallocatechin Gallate on Diethylnitrosamine-Induced Liver Tumorigenesis in Obese and Diabetic C57BL/KsJ-*db/db* Mice

Masahito Shimizu, Hiroyasu Sakai, Yohei Shirakami, et al.

Updated version	Access the most recent version of this article at: http://cancerpreventionresearch.aacrjournals.org/content/4/3/396

Cited articles	This article cites 47 articles, 12 of which you can access for free at: http://cancerpreventionresearch.aacrjournals.org/content/4/3/396.full#ref-list-1
Citing articles	This article has been cited by 2 HighWire-hosted articles. Access the articles at: http://cancerpreventionresearch.aacrjournals.org/content/4/3/396.full#related-urls

E-mail alerts	Sign up to receive free email-alerts related to this article or journal.
Reprints and Subscriptions	To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.
Permissions	To request permission to re-use all or part of this article, use this link http://cancerpreventionresearch.aacrjournals.org/content/4/3/396. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.