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Non-alcoholic steatohepatitis and preneoplastic lesions develop in the liver of obese and hypertensive rats: Suppressing effects of EGCG on the development of liver lesions

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ABSTRACT

Non-alcoholic steatohepatitis (NASH), which involves hepatic inflammation and fibrosis, is associated with liver carcinogenesis. The activation of the renin-angiotensin system (RAS), which plays a key role in blood pressure regulation, promotes hepatic fibrogenesis. In this study, we investigated the effects of (−)-epigallocatechin-3-gallate (EGCG), a major component of green tea catechins, on the development of glutathione *S*-transferase placental form (GST-P)-positive (GST-P$^+$) foci, a hepatic preneoplastic lesion, in SHRSP.Z-*Lepr*^{fa}/IzmDmcr (SHRSP-ZF) obese and hypertensive rats. Male 7-week-old SHRSP-ZF rats and control non-obese and normotensive WKY rats were fed a high fat diet and received intraperitoneal injections of carbon tetrachloride twice a week for 8 weeks. The rats were also provided tap water containing 0.1% EGCG during the experiment. SHRSP-ZF rats presented with obesity, insulin resistance, dyslipidemia, an imbalance of adipokines in the serum, and hepatic steatosis. The development of GST-P$^+$ foci and liver fibrosis was markedly accelerated in SHRSP-ZF rats compared to that in control rats. Additionally, in SHRSP-ZF rats, RAS was activated and inflammation and oxidative stress were induced. Administration of EGCG, however, inhibited the development of hepatic premalignant lesions by improving liver fibrosis, inhibiting RAS activation, and attenuating inflammation and oxidative stress in SHRSP-ZF rats. In conclusion, obese and hypertensive SHRSP-ZF rats treated with a high fat diet and carbon tetrachloride displayed the histopathological and pathophysiological characteristics of NASH and developed GST-P$^+$ foci hepatic premalignant lesions, suggesting the model might be useful for the evaluation of NASH-related liver tumorigenesis. EGCG might also be able to prevent NASH-related liver fibrosis and tumorigenesis.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD), which is strongly associated with obesity, diabetes mellitus, and the metabolic syndrome, is becoming one of the most common liver diseases worldwide. NAFLD ranges from simple steatosis to non-alcoholic steatohepatitis (NASH), which is a severe condition of inflamed fatty liver that can progress to hepatic fibrosis, cirrhosis, or even hepatocellular carcinoma (HCC) [1,2]. HCC often occurs in patients with NASH, especially in those with advanced fibrosis and cirrhosis, and the occurrence of HCC is the strongest predictor of mortality in patients with advanced fibrosis [3]. Therefore, in order to improve the prognosis of the patients with NASH, it is necessary to elucidate the pathological mechanisms implicated in the progression of liver fibrosis and HCC development. Several pathophysiological mechanisms explaining the development of HCC in NASH have been described, including the emergence of insulin resistance, induction of chronic inflammation and oxidative stress, and an imbalance of adipokines [1–6]. However, appropriate animal models to evaluate NASH-related liver fibrosis and carcinogenesis have not yet been generated.

Recently, angiotensin-II (AT-II) has been implicated as an important molecule in the progression of liver fibrosis and steatosis [7–9]. AT-II is a component of the renin-angiotensin system (RAS), a key regulator of arterial pressure, and has been shown to induce the contractility and proliferation of hepatic stellate cells (HSCs), which play a pivotal role in liver fibrogenesis [7–9]. RAS is frequently activated in patients with hepatic cirrhosis [8]. Activation of RAS has also been implicated in the etiology of hypertension, obesity, and metabolic syndrome [10]. These findings are significant when considering NASH-related liver carcinogenesis because most patients with NASH that develop HCC experience complications with obesity, diabetes, hypertension, and cirrhosis

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[11]. In addition, AT-II might play a role in the induction of oxidative stress and chronic inflammation in the liver [12,13], both of which are critically involved in the pathogenesis and progression of NASH and the related development of HCC [1–5]. These reports indicate that targeting RAS activation, which is associated with obesity and hypertension, might be an effective strategy to inhibit NASH-related liver carcinogenesis.

The SHRSP.Z-*Lepr*^{fa}/IzmDmcr (SHRSP-ZF) rat is an obese and hypertensive rat, established by crossing stroke-prone spontaneously hypertensive rats (SHRSP) with Zucker Fatty (ZF) rats [14]. SHRSP-ZF rats inherit the leptin receptor *OB-ob* gene mutation found in ZF rats and become obese while developing hypertension. Therefore, the phenotype resembles that of human metabolic syndrome. The rats may thus be a useful tool for investigating the molecular mechanisms underlying metabolic syndrome [15,16]. We therefore considered that appropriate treatment(s) to the SHRSP-ZF rats enable us to establish a novel animal model of NASH and NASH-related hepatocarcinogenesis that mimics those of humans and to use as a preclinical animal model for chemoprevention studies for the diseases.

In the present study, we aimed to create a new NASH-related liver tumorigenesis rat model that appropriately reflects the pathological conditions of human NASH by using SHRSP-ZF rats. We also investigated the potential preventive effects of (−)-epigallocatechin-3-gallate (EGCG), a green tea catechin (GTC), on liver fibrosis, steatosis, and tumorigenesis using this rodent model because green tea is considered to prevent metabolic disorders, including obesity, insulin resistance, hypertension, and NAFLD [17–19], as well as possesses anticancer and cancer chemopreventive properties in various organs, including the liver [20–23]. Glutathione *S*-transferase placental form (GST-P)-positive (GST-P$^+$) foci are frequently used as an indicator of preneoplastic lesions for HCC of rats, since this biomarker shows good correlations with long term carcinogenicity results [24]. We evaluated liver tumorigenesis and chemopreventive efficacy of EGCG in the SHRSP-ZF rats using GST-P$^+$ foci as a biomarker.

2. Materials and methods

2.1. Animals and chemicals

Six-week-old male SHRSP-ZF rats and control Wister Kyoto (WKY) rats, which are normotensive and do not present with obesity, were obtained from Japan SLC (Shizuoka, Japan) and humanely maintained at Gifu University Life Science Research Center in accordance with the Institutional Animal Care Guidelines. High-fat diet 32 (HFD, 507.6 kcal/100 g) with 56.7% fat derived calories was purchased from CLEA Japan (Tokyo, Japan). Carbon tetrachloride (CCl_4) was purchased from Sigma (St. Louis, MO, USA). EGCG was obtained from Mitsui Norin (Tokyo, Japan).

2.2. Experimental procedure

In a preliminary study, we confirmed that the development of preneoplastic lesions, GST-P$^+$ foci, was observed in the liver of WKY and SHRSP-ZF rats only when they were treated with both HFD and CCl_4 (data not shown). Therefore, all rats were fed a pelleted HFD throughout the experiment and received CCl_4 in the present study. After 1 week of acclimatization, 20 WKY rats (Groups 1 and 2; 10 rats for each group) and 20 SHRSP-ZF rats (Groups 3 and 4; 10 rats for each group) were randomly divided into 2 groups. All rats received an intraperitoneal injection of CCl_4 (0.5 mL/kg body weight) twice a week for 8 weeks. At the start of the intraperitoneal injections, the rats in Groups 2 and 4 were provided tap water containing 0.1% EGCG, while the rats in Groups 1 and 3 were provided tap water throughout the experiment. The concentration of EGCG (0.1%), which was established according to the findings of previous chemopreventive studies [22,23] was within the physiological range observed in humans after daily intake of GTCs on a per unit body weight basis [25]. At the end of the experiment (15 weeks of age), all rats were killed by CO_2 asphyxiation, and the development of hepatic steatosis, fibrosis, and GST-P$^+$ foci was determined.

2.3. Histopathological and immunohistochemical examinations

Maximum sagittal sections of 3 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 conventional histopathology or with Azan stain to observe liver fibrosis [26]. The histological features of the livers were evaluated using the NAFLD activity score (NAS) system [27]. The immunohistochemistry of α-smooth muscle actin (α-SMA) [26] and GST-P [28] was performed using primary anti-α-SMA (DAKO, Glostrup, Denmark) and anti-GST-P (MBL, Nagoya, Japan) antibodies, respectively, by using paraffin-embedded sections. In order to evaluate the oxidative stress and lipid peroxidation in the liver, immunohistochemical staining for 8-hydroxy-2′-deoxyguanosine (8-OHdG, NIKKEN SEIL, Shizuoka, Japan) and 4-hydroxy-2′-nonenal (4-HNE, NIKKEN SEIL) of paraffin-embedded sections was performed. Immunohistochemical staining for Mac-1 (Abcam, Cambridge, MA, USA) was also performed on the paraffin-embedded sections to evaluate the infiltration of macrophages in the liver. The Azan- and α-SMA-positive areas were quantified using BZ-Analyzer-II software (KEYENCE, Osaka, Japan) [29]. GST-P$^+$ foci, which consisted of 3 or more positive cells, were counted as hepatic preneoplastic lesions, as previously described [30], and its multiplicity was assessed on a unit area basis (per cm^2). The assessment for GST-P$^+$ foci development and the NAS scoring system were blinded from each other.

2.4. RNA extraction and quantitative real-time reverse transcription-polymerase chain reaction analysis

Total RNA was isolated from the livers of experimental rats using the RNAqueous-4PCR kit (Ambion Applied Biosystems, Austin, TX, USA). 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 *tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, monocyte chemoattractant protein-1 (MCP-1), plasminogen activator inhibitor-1 (PAI-1), transforming growth factor (TGF)-β1, α-SMA, procollagen-1, tissue inhibitor of metalloproteinases (TIMP)-1, TIMP-2, matrix metalloproteinases (MMP)-2, MMP-9, angiotensin-converting enzyme (ACE), AT-II type 1 receptor (AT-1R), glutathione peroxidase (GPx), catalase (CAT),* and *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* genes. The sequences of *TNF-α, IL-1β, IL-6, MCP-1, PAI-1, TIMP-1, TIMP-2, MMP-2, MMP-9, ACE,* and *AT-1R* primers, which were obtained from Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/), are shown in Supplemental Table S1. The sequences of other primers are described in a previous report [31]. Each sample was analyzed on a LightCycler Nano (Roche Diagnostics, GmbH, Mannheim, Germany) with FastStart Essential DNA Green Master (Roche Diagnostics). Parallel amplification of *GAPDH* was used as the internal control.

2.5. Protein extraction and western blot analysis

Total protein was extracted from the livers of experimental rats and equivalent amounts of proteins (20 μg/lane) were examined by western blot analysis [23]. The primary antibody for cytochrome P450 2E1 (CYP2E1) was purchased from Abcam. Primary antibodies for c-Jun NH2-terminal kinase (JNK), phosphorylated JNK (p-JNK), and GAPDH were obtained from Cell Signaling Technology (Beverly, MA, USA). The antibody to GAPDH served as the loading control.

2.6. Clinical chemistry

The blood samples collected from the inferior vena cava of the rats at the time of killing after 6 h of fasting were used for chemical analyses. The serum levels of TNF-α (R&D Systems, Minneapolis, MN, USA), IL-6 (R&D Systems), insulin (Shibayagi, Gunma, Japan), glucose (BioVision Research Products, Mountain View, CA, USA), adiponectin (Shibayagi), leptin (Shibayagi), total cholesterol (Wako Pure Chemical, Osaka, Japan), triglyceride (Wako Pure Chemical), non-esterified fatty acid (NEFA) (Wako Pure Chemical), and AT-II (USCN Life Science Inc, Wuhan, China) were determined by enzyme immunoassay according to the manufacturers' protocols. The serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured using a standard clinical automatic analyzer (type 7180; Hitachi, Tokyo, Japan).

2.7. Hepatic hydroxyproline analysis

The hepatic hydroxyproline content (μmol/g wet liver) was quantified colorimetrically in duplicate samples from approximately 200 mg wet-weight of liver tissues [32].

2.8. Oxidative stress analysis

Serum hydroperoxide levels, one of the markers for oxidative stress, were determined using the derivatives of reactive oxygen metabolites (d-ROM) test (FREE Carpe Diem; Diacron s.r.l., Grosseto, Italy). After equalizing the protein contents, hepatic levels of malondialdehyde (MDA) were evaluated using an MDA assay kit (Northwest Life Science Specialties, Vancouver, WA, USA).

Table 1
Body, liver, and adipose tissue weights and BMI of the experimental rats.

Group no.	Strain	EGCG	No. of rats	Body weight (g)	Relative organ weight (g/100 g body weight)		BMI[b]
					Liver	Adipose[a]	
G1	WKY	–	10	312.5 ± 13.3[c]	4.1 ± 1.0	1.9 ± 0.4	6.0 ± 0.4
G2	WKY	+	10	296.8 ± 19.4	3.7 ± 0.2	2.0 ± 0.2	6.0 ± 0.3
G3	SHRSP-ZF	–	10	352.9 ± 37.9[d]	5.6 ± 0.6[d]	2.8 ± 0.2[d]	8.3 ± 0.9[d]
G4	SHRSP-ZF	+	10	421.1 ± 38.7[e,f]	6.4 ± 0.4[e]	2.8 ± 0.1[e]	9.4 ± 0.7[e,f]

[a] White adipose tissue of the periorchis and retroperitoneum.
[b] Body mass index.
[c] Mean ± SD.
[d] Significantly different from group 1 by Tukey–Kramer multiple comparison test ($P < 0.05$).
[e] Significantly different from group 2 by Tukey–Kramer multiple comparison test ($P < 0.05$).
[f] Significantly different from group 3 by Tukey–Kramer multiple comparison test ($P < 0.01$).

2.9. Statistical analysis

All data are presented as mean ± SD and were analyzed using the GraphPad In-Stat software program version 3.05 (GraphPad Software, San Diego, CA) for Macintosh. One-way analysis of variance (ANOVA) was used to make comparison between the groups. If the ANOVA analysis indicated significant differences, the Tukey–Kramer multiple comparisons test was performed to compare the mean values among the groups. The differences were considered significant when the two-sided P value was less than 0.05.

3. Results

3.1. General observations

The body weights, relative weights of liver and adipose tissues, and body mass index (BMI) of the SHRSP-ZF rats were significantly higher than those of the WKY rats, regardless of EGCG treatment (Table 1; $P < 0.05$). In SHRSP-ZF rats, the body weights and BMI of the EGCG-treated rats were significantly higher than those of untreated rats ($P < 0.01$), suggesting that EGCG might prevent body weight loss caused by liver fibrosis. During the experiment, EGCG in the drinking water did not cause any clinical symptoms for toxicity. Histopathological examinations also revealed the absence of toxicity from EGCG in the liver, kidney, and spleen (data not shown).

3.2. Effects of EGCG on the development of hepatic preneoplastic lesions and histopathology in the experimental rats

Irrespective of the rat strain, GST-P$^+$ foci were observed in the livers of rats from all groups at the termination of the experiment (Fig. 1A). However, the number of foci was significantly increased, by approximately 5.2-fold, in SHRSP-ZF rats compared to that in WKY rats (Fig. 1B; $P < 0.001$), indicating that obesity and hypertension play a critical role in accelerating the development of hepatic preneoplastic lesions. On the other hand, EGCG treatment significantly inhibited the development of GST-P$^+$ foci in obese and hypertensive SHRSP-ZF rats ($P < 0.001$).

Steatosis with ballooning and/or Mallory-Deng body (Fig. 1C and D), and the infiltration of macrophages (Fig. 1E), which are a recognized feature of alcoholic hepatitis and NASH [27], were observed in the liver of both strains of rats that received CCl_4. However, the NAS scores, which reflect the sum of steatosis, hepatocyte ballooning, and lobular inflammation [27], were significantly higher in the SHRSP-ZF rats than in the WKY rats (Fig. 1F; $P < 0.01$). When given EGCG, the NAS score was improved in SHRSP-ZF rats ($P < 0.01$).

3.3. Effects of EGCG on liver fibrosis in the experimental rats

Azan-stained sections indicated that SHRSP-ZF and WKY rats developed liver fibrosis after CCl_4 injection. However, the degree of fibrosis was more severe in SHRSP-ZF rats; densitometric analysis showed that the hepatic fibrosis area in SHRSP-ZF rats was significantly larger than that in WKY rats (Fig. 2A; $P < 0.001$). Densitometric analysis of α-SMA immunohistochemistry also showed that the α-SMA-immunoreactive areas, which reflect the activation of HSCs, were remarkably increased in the livers of SHRSP-ZF rats in comparison with those in the livers of WKY rats (Fig. 2B; $P < 0.001$). However, administration of EGCG through drinking water significantly improved CCl_4-induced liver fibrosis and inhibited the activation of HSCs in SHRSP-ZF rats (Fig. 2A and B; $P < 0.001$).

Similar findings were observed in the measurements of the hepatic hydroxyproline contents. The amount of hydroxyproline in the liver, which was approximately 7.2-fold higher in SHRSP-ZF rats than in WKY rats ($P < 0.001$), decreased significantly after EGCG treatment (Fig. 2C; $P < 0.01$). Moreover, quantitative real-time RT-PCR analysis revealed that, in the livers of SHRSP-ZF rats, EGCG significantly decreased the expression levels of *MMP-2*, *MMP-9*, *TIMP-1*, *TIMP-2*, *α-SMA*, *procollagen-1*, *TGF-β1*, and *PAI-1* mRNA ($P < 0.05$), all of which were remarkably higher in SHRSP-ZF rats than in WKY rats (Fig. 2D; $P < 0.05$).

3.4. Effects of EGCG on serum levels of AT-II and hepatic expression of ACE and AT-1R mRNA in the experimental rats

Hyperactivity of RAS is closely associated with liver fibrosis and carcinogenesis [8,33]. Therefore, the serum levels of AT-II and the expression levels of RAS components, including *ACE* and *AT-1R* mRNA in the liver, were investigated. The serum level of AT-II was markedly elevated in SHRSP-ZF rats compared to that in WKY rats ($P < 0.001$), but was significantly decreased by EGCG treatment (Fig. 3A; $P < 0.05$). In SHRSP-ZF rats, there was a marked increase in the expression levels of *ACE* and *AT-1R* mRNA in the liver ($P < 0.05$); however, EGCG significantly decreased the expression levels of these mRNA (Fig. 3B; $P < 0.05$).

3.5. Effects of EGCG on oxidative stress, lipid peroxidation in the liver, and hepatic expression of CYP2E1, JNK, and p-JNK proteins in the experimental rats

Hepatic oxidative stress and lipid peroxidation are implicated in the hepatic fibrogenesis, progression of fatty livers to NASH, and development of HCC [4,6]. Therefore, the levels of oxidative stress and antioxidant biomarkers in the experimental rats were next assessed. SHRSP-ZF rats showed a significant increase in serum

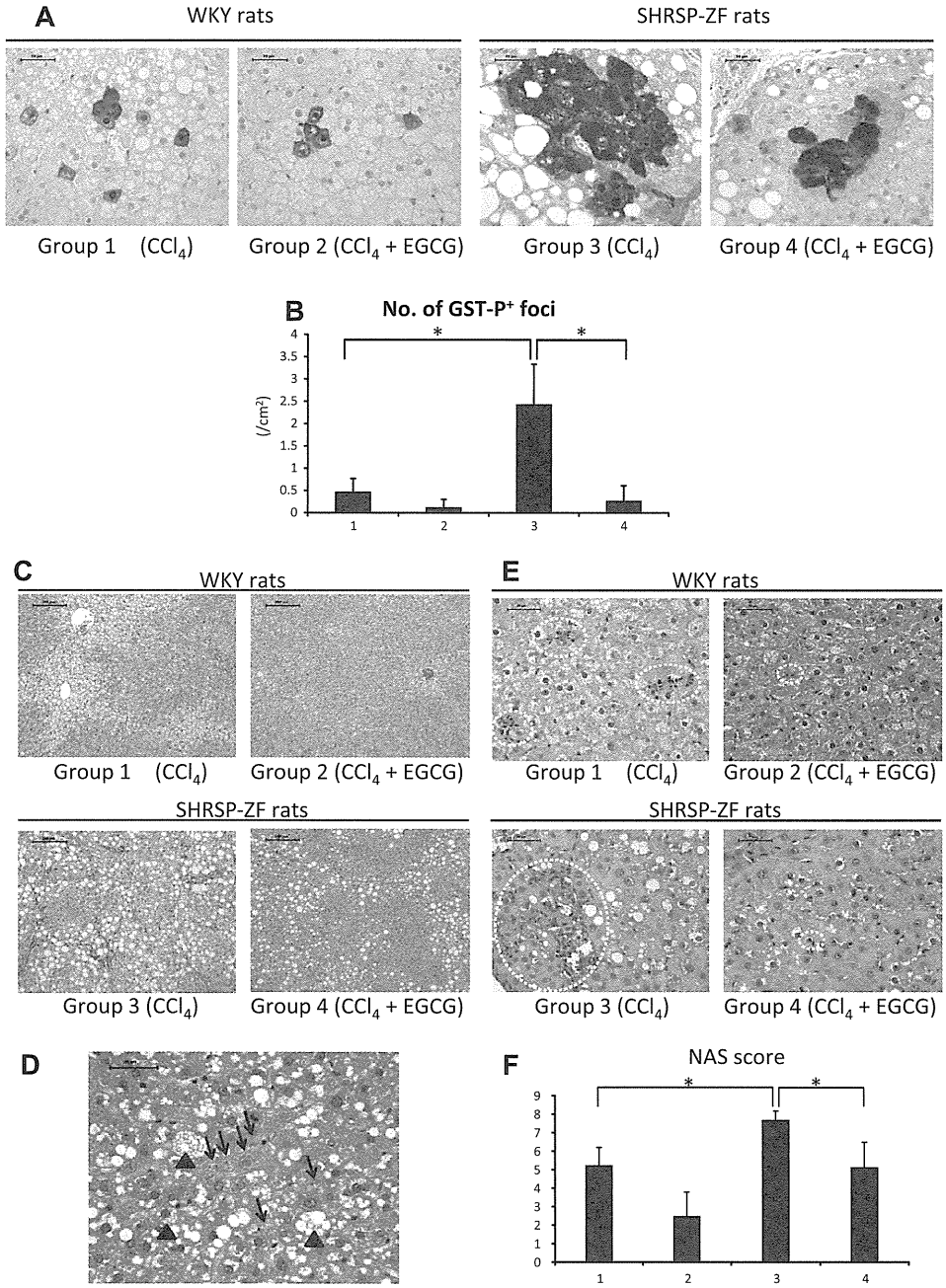

Fig. 1. Effects of EGCG on the development of GST-P$^+$ foci and histopathology in the livers of the experimental rats. (A) Representative photomicrographs of GST-P$^+$ foci and (B) the average number of GST-P$^+$ foci that developed in the livers of the experimental rats. Group 1: WKY rats treated without EGCG, Group 2: WKY rats treated with EGCG, Group 3: SHRSP-ZF rats treated without EGCG, and Group 4: SHRSP-ZF rats treated with EGCG. (C and D) Histopathology of the livers of the experimental rats. H&E staining of liver paraffin sections show steatosis with fibrosis and fatty degeneration in the WKY and SHRSP-ZF rats that were fed HFD and received CCl_4. (D) High magnification of view shows liver cell ballooning (arrow heads) and Mallory-Deng body (arrows) in the liver of a SHRSP-ZF rat from Group 3. (E) The results of the immunohistochemical analysis of Mac-1 in the livers of the experimental rats. Infiltration of macrophages is indicated with circular broken lines. (F) The NAS score (steatosis, inflammation, and ballooning) was determined based on the histopathological analysis. Bars are (A and C) 200 μm and (D and E) 50 μm. The values are expressed as mean ± SD. $^*P < 0.001$.

d-ROM levels, which reflect serum hydroperoxide levels ($P < 0.001$), but this increase was significantly attenuated by EGCG treatment (Fig. 4A; $P < 0.05$). The increased levels of hepatic MDA, a marker of hepatic lipid peroxidation, in SHRSP-ZF rats ($P < 0.05$) were also reduced by EGCG treatment (Fig. 4B; $P < 0.05$). These findings are consistent with the results of immunohistochemical analysis for 8-OHdG, a product of hydroxyl radical-induced oxidative damage in DNA, and 4-HNE, a marker of lipid peroxidation.

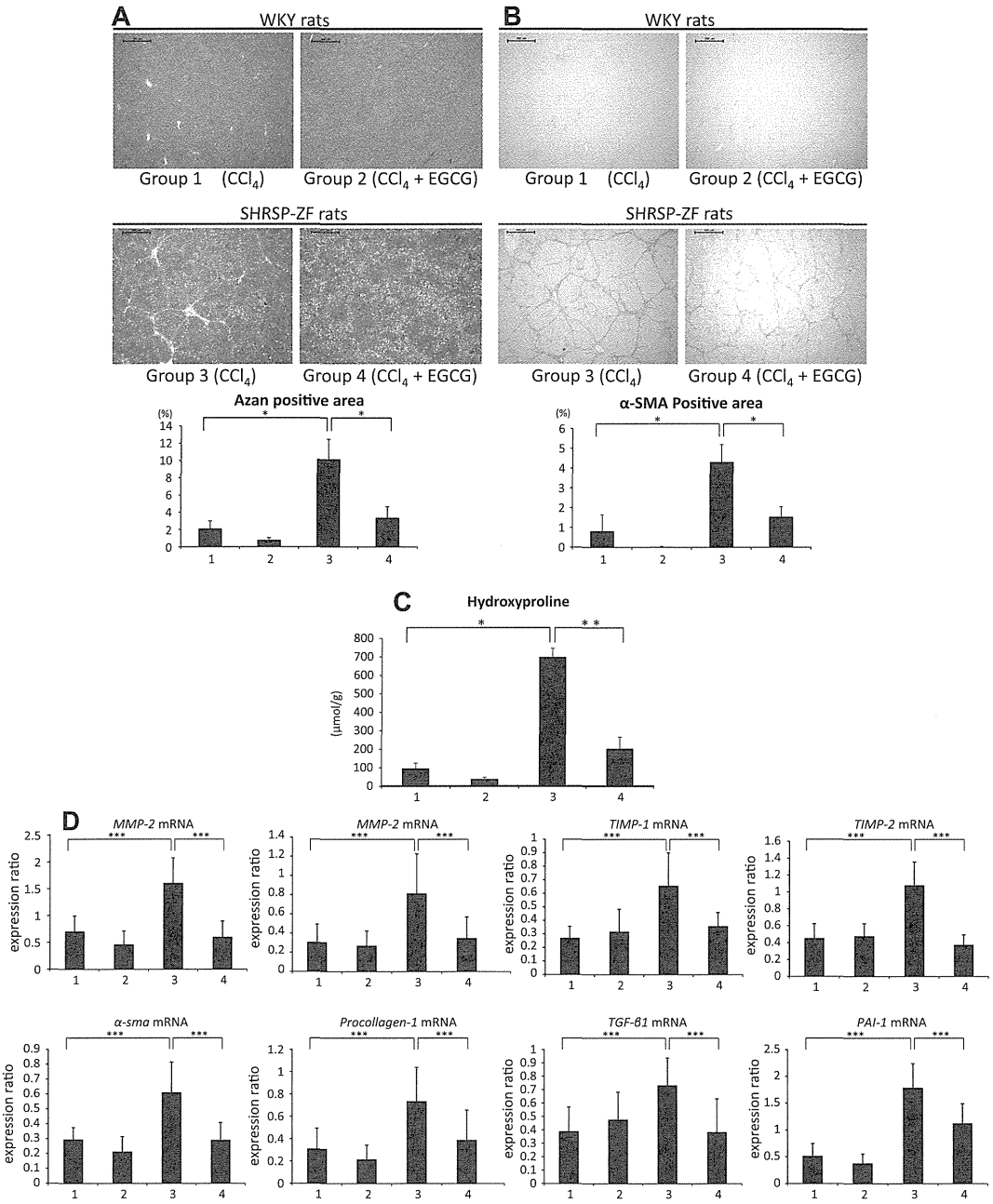

Fig. 2. Effects of EGCG on hepatic fibrosis in the experimental rats. (A) Representative photomicrographs of liver sections stained with Azan stain to show fibrosis (upper panels). The hepatic fibrosis area was evaluated by Azan stain (lower panel). (B) Immunohistochemical detection of α-SMA expression in the livers of the experimental rats (upper panels). The α-SMA-positive area, which shows the activation of HSCs, was evaluated using an image analyzer (lower panel). (C) The hepatic hydroxyproline content was quantified colorimetrically. (D) Total RNA was isolated from the livers of experimental rats, and the expression levels of MMP-2, MMP-9, TIMP-1, TIMP-2, α-SMA, TGF-β1, procollagen-1, and PAI-1 mRNA were examined by quantitative real-time RT-PCR by using specific primers. Bars are 200 μm. The values are expressed as mean ± SD. $^{*}P < 0.001$, $^{**}P < 0.01$, $^{***}P < 0.05$.

The expression levels of 8-OHdG and 4-HNE proteins were markedly increased in the hepatocytes of SHRSP-ZF rats, but they were decreased by EGCG treatment (Fig. 4C). Furthermore, the increased levels of hepatic CYP2E1 and p-JNK proteins, both of which are critically important in HFD-induced NASH development by promoting oxidative stress and inflammation [34,35] in SHRSP-ZF rats were also decreased by EGCG treatment (Fig. 4D). On the other hand, the reduced expression levels of *GPx* and *CAT* mRNA, which encode antioxidant enzymes, in SHRSP-ZF rats ($P < 0.05$) were effectively restored by EGCG treatment (Fig. 4E; $P < 0.05$).

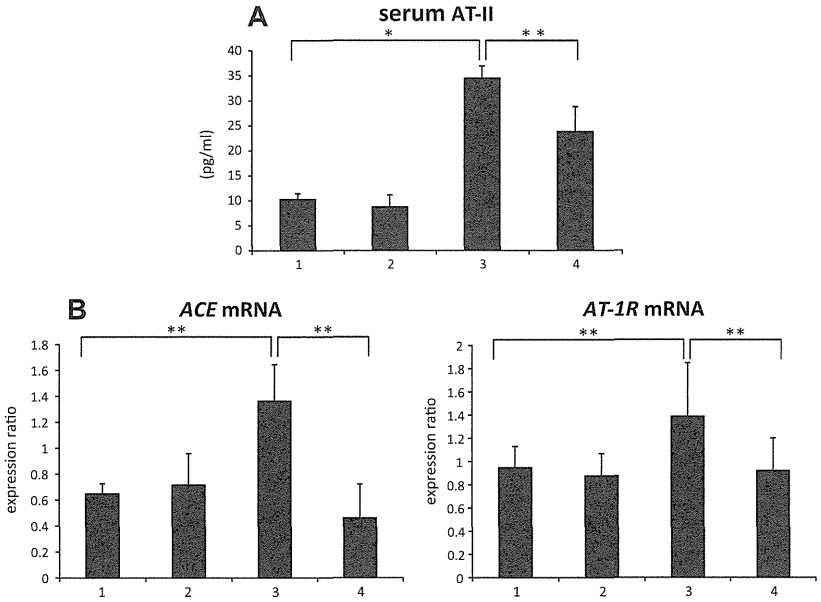

Fig. 3. Effects of EGCG on renin-angiotensin system in the experimental rats. (A) The serum concentrations of AT-II were measured using enzyme immunoassay. (B) The expression levels of ACE and AT-1R mRNA in the livers of the experimental rats were examined by quantitative real-time RT-PCR by using specific primers. The values are expressed as mean ± SD. $^{*}P < 0.001$, $^{**}P < 0.05$.

3.6. Effects of EGCG on serum levels of TNF-α and IL-6 and hepatic expression of TNF-α, IL-6, IL-1β, and MCP-1 mRNA in the experimental rats

Chronic inflammation plays a critical role in the progression of liver fibrosis and subsequent HCC development [5]. Therefore, the levels of inflammatory mediators, including TNF-α, IL-6, IL-1β, and MCP-1, were investigated. The serum levels of TNF-α and IL-6 in SHRSP-ZF rats were significantly elevated relative to those in WKY rats (Fig. 5A; $P < 0.05$). There was also a marked increase in the expression levels of *TNF-α*, *IL-6*, *IL-1β*, and *MCP-1* mRNA in the livers of SHRSP-ZF rats (Fig. 5B; $P < 0.05$). Although EGCG treatment did not significantly affect the serum levels of TNF-α and IL-6 in both SHRSP-ZF and WKY rats (Fig. 5A), the treatment significantly decreased the hepatic expression levels of *TNF-α*, *IL-6*, *IL-1β*, and *MCP-1* mRNA in SHRSP-ZF rats (Fig. 5B, $P < 0.05$).

3.7. Effects of EGCG on serum parameters in the experimental rats

Irrespective of EGCG treatment, the serum levels of AST, ALT, total cholesterol, NEFA, and triglycerides in SHRSP-ZF rats were significantly higher than those in WKY rats (Table 2; $P < 0.05$). The serum levels of glucose and insulin increased significantly, while the value of QUICKI, a useful index of insulin sensitivity [36], decreased ($P < 0.05$). The serum levels of leptin in SHRSP-ZF rats were significantly elevated relative to those in WKY rats, but the levels of adiponectin were lower ($P < 0.05$). Among the parameters elevated in SHRSP-ZF rats, only the serum level of NEFA was significantly suppressed by EGCG treatment ($P < 0.05$). These findings suggest that, in comparison to the improvement of insulin resistance and adipokine imbalance, reduction of oxidative stress and attenuation of inflammation in the liver (Figs. 4 and 5) are more critical mechanisms of EGCG that prevented the early phase of NASH-related liver carcinogenesis in the present study.

4. Discussion

In order to develop an effective strategy for the prevention of NASH-related liver tumorigenesis, there is a critical need to establish an appropriate rodent model that displays the histopathological and pathophysiological characteristics of NASH. The present study provides the first evidence that SHRSP-ZF rats, which present with obesity, diabetes, and hypertension and thus mimic human metabolic syndrome [14,15], more readily develop hepatic preneoplastic lesions, GST-P^{+} foci, than non-obese and normotensive WKY rats when the rats were fed HFD and received CCl_4 injections. The results of the present study clearly indicate that early phase of hepatic tumorigenesis is associated with accelerated steatosis, liver fibrosis, chronic liver damage, presence of insulin resistance, imbalance of adipokines and induction of chronic inflammation and oxidative stress. Because these pathophysiological conditions are critically involved in the progression of NASH and its related liver tumorigenesis [1–5], we propose that our new model using SHRSP-ZF rats might be useful for analyzing the mechanisms of NASH-related liver tumorigenesis and evaluating the efficacy of specific agents that can prevent such tumorigenesis.

One of the limitations in the current study is that we did not observe hepatocellular neoplasms. This might be associated with the duration of the experiment (8 weeks), which was insufficient to develop hepatic tumors. Therefore, future study should recruit longer-term experiments to see that HFD- and CCl_4-treated SHRSP-ZF rats develop hepatocellular neoplasms. Long-term experiments are also useful for evaluating whether the alteration of hepatic gene expression occurred in the present short-term study contribute to the development of hepatocellular neoplasms practically. In addition, it remains unclear whether, not only obesity, but also hypertension actually plays a critical role in the early events of liver carcinogenesis. There are no previous studies that have evaluated the effect of HFD and CCl_4 treatment in hypertensive SHRSP rats as well as in obese ZF rats. Therefore, in order to dissect the effect of hypertension or obesity in liver carcinogenesis,

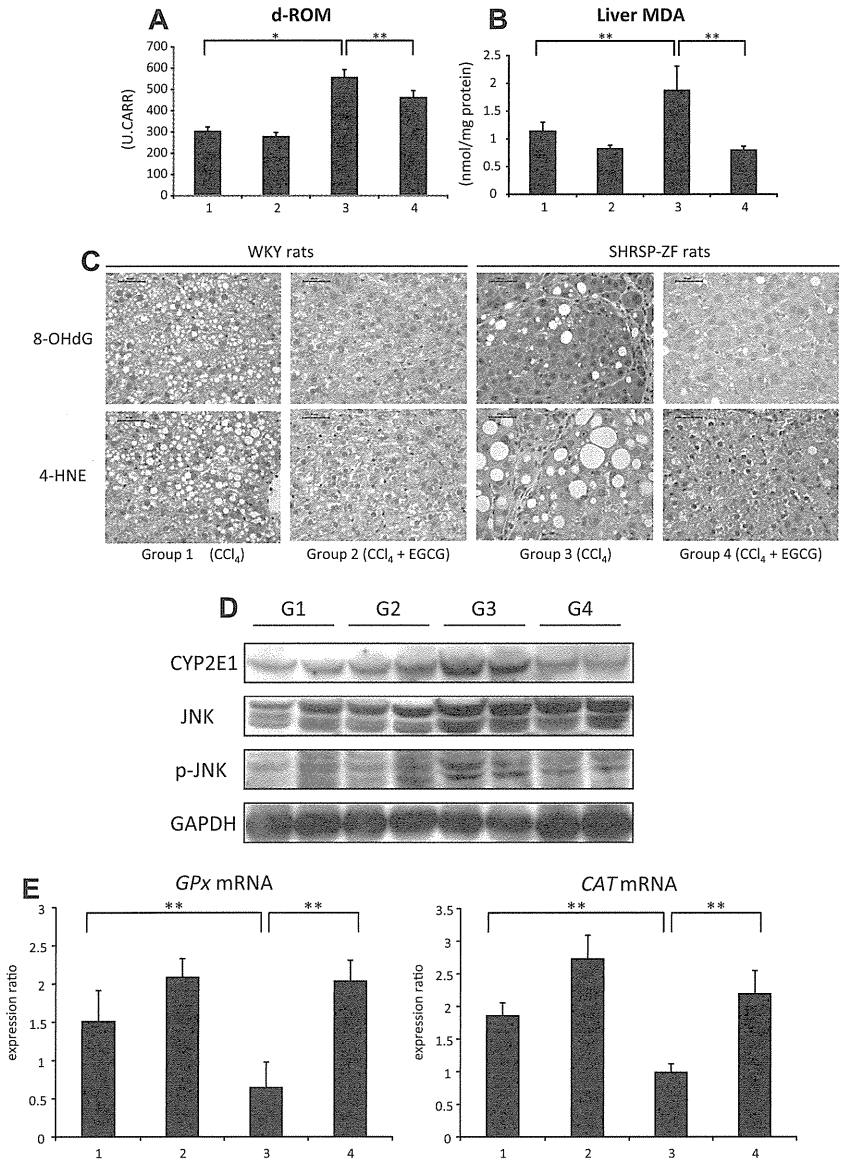

Fig. 4. Effects of EGCG on the serum levels of d-ROM, hepatic concentration of MDA, hepatic expression levels of 8-OHdG, 4-HNE, CYP2E1, JNK, and p-JNK proteins, and hepatic expression levels of GPx and CAT mRNA in the experimental rats. (A) Hydroperoxide levels in the serum were determined by the d-ROM test. (B) The hepatic concentration of MDA was measured by enzyme immunoassay. (C) The results of the immunohistochemical analyses of 8-OHdG and 4-HNE in the livers of the experimental rats. (D) Total proteins were extracted from the livers of the experimental rats and the expression levels of CYP2E1, JNK, and p-JNK proteins were examined by western blot analysis. GAPDH antibody served as the loading control. (E) Total RNA was isolated from the livers of experimental rats, and the expression levels of GPx and CAT mRNA were examined by quantitative real-time RT-PCR by using specific primers. Bars are 50 μm (C). The values are expressed as mean ± SD. $^{*}P < 0.001$, $^{**}P < 0.05$.

additional studies that examine the effects of HFD and CCl_4 treatment in SHRSP rats and ZF rats should be conducted. On the other hand, this study aimed to compare the development of fibrogenesis and preneoplastic lesions (GST-P^+ foci) between the SHRSP-ZF and WKY rats in order to establish NASH-associated liver carcinogenesis. Because GST-P^+ foci are generally accepted as precursor or preneoplastic lesions for HCC in rodents [28,30,37], our findings suggest high susceptibility of the obese and hypertensive SHRSP-ZF rats to hapetocarcinogeneis.

What key mechanism accelerates liver fibrosis and tumorigenesis in SHRSP-ZF rats? We presume that activation of RAS caused by obesity and hypertension is critically involved in such disorders in SHRSP-ZF rats because RAS appears to play a major role in liver fibrosis [38]. AT-II induces the fibrotic effect in activated HSCs by stimulating TGF-β1 expression and increasing collagen synthesis in the liver through the activation of its receptor, AT-1R [8,9,38]. Activated HSCs, which highly express AT-1R, are capable of generating AT-II, suggesting that AT-II can act in an autocrine/paracrine manner in HSCs when liver fibrosis progresses [8]. On the other hand, blocking the generation of AT-II and/or its binding to AT-1R attenuates fibrosis development in experimental rodent models of chronic liver injury [39]. Moreover, the potential beneficial abil-

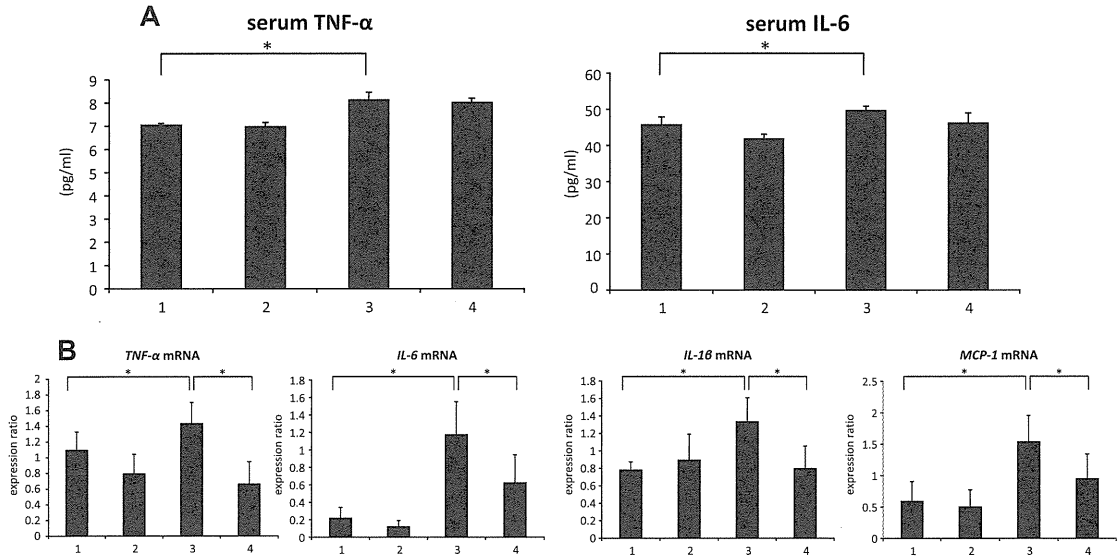

Fig. 5. Effects of EGCG on the serum levels of TNF-α and IL-6 and the expression levels of TNF-α, IL-6, IL-1β, and MCP-1 mRNA in the livers of the experimental rats. (A) The serum concentrations of TNF-α and IL-6 were measured by enzyme immunoassay. (B) Total RNA was isolated from the livers of experimental rats, and the expression levels of TNF-α, IL-6, IL-1β, and MCP-1 mRNA were determined by quantitative real-time RT-PCR by using specific primers. The values are expressed as mean ± SD. $^{*}P < 0.05$.

Table 2
Serum parameters in the experimental rats.

	Group 1	Group 2	Group 3	Group 4
AST (IU/l)	166.8 ± 16.9[a]	140.5 ± 23.6	325.3 ± 45.5[b]	293.0 ± 46.9[c]
ALT (IU/l)	35.5 ± 2.1	36.5 ± 5.4	183.8 ± 42.2[b]	219.3 ± 41.7[c]
Glucose (mg/dl)	106.7 ± 7.2	105.3 ± 4.6	135.1 ± 3.8[b]	127.8 ± 5.3[c]
Insulin (μIU/ml)	25.5 ± 5.4	50.6 ± 8.8	183.4 ± 61.3[b]	223.1 ± 37.5[c]
QUICKI	0.292 ± 0.009	0.269 ± 0.004	0.226 ± 0.008[b]	0.225 ± 0.002[c]
Adiponectin (ng/ml)	52.9 ± 1.9	52.2 ± 0.4	35.2 ± 5.8[b]	35.4 ± 4.0[c]
Leptin (pg/ml)	47.4 ± 4.7	48.4 ± 3.2	400.4 ± 7.3[b]	398.3 ± 5.8[c]
Total cholesterol (mg/dl)	98.2 ± 6.7	93.2 ± 5.0	151.8 ± 9.6[b]	149.8 ± 5.1[c]
NEFA (mEq/L)	0.311 ± 0.038	0.267 ± 0.035	0.698 ± 0.059[b]	0.577 ± 0.046[c,d]
Triglyceride (mg/dl)	53.7 ± 8.1	46.7 ± 8.5	139.9 ± 10.1[b]	128.8 ± 10.9[c]

[a] Mean ± SD.
[b] Significantly different from group 1 by Tukey–Kramer multiple comparison test ($P < 0.05$).
[c] Significantly different from group 2 by Tukey–Kramer multiple comparison test ($P < 0.05$).
[d] Significantly different from group 3 by Tukey–Kramer multiple comparison test ($P < 0.05$).

ity of RAS inhibitors in the attenuation of liver fibrosis in patients with NASH has been shown in clinical trials [40]. Therefore, in the present study, activation of RAS plays a pivotal role in the progression of liver fibrosis in obese and hypertensive SHRSP-ZF rats. EGCG inhibits this fibrogenesis, at least in part, by targeting RAS activation because this agent decreases serum levels of AT-II and suppresses the expression of *ACE* and *AT-1R* mRNA in the liver of these rats. The inhibition of liver fibrosis is significant when considering the chemoprevention of HCC because the risk of liver carcinogenesis increases along with the progression of liver fibrosis [41].

In the liver, RAS is also involved in chronic inflammation and oxidative stress, both of which play a critical role in the progression of fibrosis and subsequent carcinogenesis [8,33]. Administration of AT-II to rats induces HSCs activation, hepatic inflammation, oxidative stress, and lipid peroxidation [42]. Increased systemic AT-II also augments hepatic fibrosis and promotes inflammation and oxidative stress in rats undergoing biliary fibrosis [43]. AT-II stimulates the secretion of inflammatory cytokines such as TNF-α and MCP-1 [44], both of which are involved in the progression of NASH [2], suggesting that targeting RAS might be an effective way to attenuate chronic inflammation and reduce oxidative stress in NASH. AT-1R blockade suppresses HSCs activation, inhibits TNF-α expression, and reduces oxidative stress in rats fed a methionine-choline–deficient diet [39]. The specific delivery of an AT-1R blocker to activated HSCs also reduces inflammation and advanced liver fibrosis in rats [45]. Therefore, consistent with these reports [39,45], EGCG might also prevent liver fibrosis and subsequent tumorigenesis in obese and hypertensive rats by reducing chronic inflammation, systemic oxidative stress, and liver peroxidation, which were induced by RAS activation in the present study. In particular, the effects of EGCG on suppression of the elevated CYP2E1 protein in SHRSP-ZF rats is significant because CYP2E1, which is increased by HFD feeding, is critical in NASH development by promoting oxidative stress, lipid peroxidation, and inflammation [34,35].

Numerous clinical trials have been conducted to develop a therapy that is of proven benefit for NASH; however, no optimal treatment for this disease has yet been found. One of the most practical approaches to treat NASH is targeting insulin resistance and oxidative stress, both of which are implicated as key factors contributing to hepatic injury in patients with NASH [2]. A meta-analysis has

shown that thiazolidinediones, insulin sensitizers regulating glucose metabolism, improve steatosis and serum ALT levels in these patients [46]. In a recent randomized trial with NASH patients, treatment with vitamin E, an antioxidant, also reduced steatosis, lobular inflammation, and serum ALT and AST levels [47]. In the present study, EGCG significantly prevented NASH-related liver fibrosis and tumorigenesis, at least in part, by reducing oxidative stress. Moreover, EGCG also suppresses obesity-related liver and colorectal carcinogenesis by improving hyperinsulinemia [21,23]. The effects of GTCs, whereby they suppress metabolic syndrome, have also been investigated in laboratory animal, epidemiological, and intervention studies [17–19]. These reports [18,19,21,23], together with our findings described here, strongly suggest that GTCs may be useful for preventing the progression of NASH-related liver tumorigenesis, which is associated with oxidative stress and insulin resistance.

Finally, it should be mentioned that the beneficial effects of GTCs have been reported in clinical trials. Supplementation with GTCs can significantly prevent the development of both colorectal adenomas and prostate cancers without causing adverse effects [48,49]. These findings are significant because there are risks associated with medications that are expected to improve NASH, such as weight gain with thiazolidinediones and cardiovascular events and hemorrhagic stokes with vitamin E [46,47]. In summary, our data showed for the first time that liver fibrosis and the development of hepatocellular preneoplastic lesions (GST-P$^+$ foci) are significantly enhanced in obese and hypertensive SHRSP-ZF rats treated with HFD and CCl_4, which have characteristics similar to human NASH. Administration of EGCG effectively prevents liver fibrosis and early stage of hepatocarcinogenesis in these rats by targeting RAS activation and the subsequent inflammation and oxidative stress. Previous rodents studies have shown that GTCs prevent hypertension and target organ damage induced by AT-II through the reduction of oxidative stress [50,51]. GTCs also have a significant inhibitory effect on the activity of ACE and this might be associated with the suppression of high blood pressure in a clinical trial [52]. Although we did not measure the blood pressure of experimental rats in the present study, the results from both experimental and clinical studies [50–52], together with those of present study, strongly indicate the possibility of GTCs, including EGCG, to inhibit RAS activation and to decrease blood pressure subsequently.

In conclusion, our model could be a good option, allowing researchers to study not only the mechanisms involved in NASH-associated hepatocarcinogenesis and the early events involved in tumor formation, but also approaches to HCC prevention in NASH patients focusing on the molecular regulators of the disease. In addition, use of EGCG can improve the NAS score, reduce oxidative stress, and also attenuate chronic inflammation. EGCG therapy represents a potential new strategy for preventing the development of hepatic fibrosis and neoplasm in NASH patients.

5. Conflict of Interest

None declared.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.canlet.2013.08.031.

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Hepatology Research

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Special Report

Nutritional status and quality of life in current patients with liver cirrhosis as assessed in 2007–2011

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Aim: Current guidelines recommended adequate nutritional support for patients with liver cirrhosis to improve clinical outcome and quality of life (QOL). However, these evidences were obtained more than 10 years ago when malnutrition prevailed. In recent years, the impact of obesity on liver damage and carcinogenesis has grown. We attempted to elucidate the nutritional state and QOL in present cirrhotics.

Methods: A research group supported by the Ministry of Health, Labor and Welfare of Japan recruited 294 cirrhotics between 2007 and 2011. Subjects comprised 171 males and 123 females, 158 of whom had hepatocellular carcinoma (HCC) and Child–Pugh grades A : B : C were 154:91:49. Anthropometry, blood biochemistry and indirect calorimetry were conducted, and QOL was measured using Short Form-8.

Results: The mean body mass index (BMI) of all patients was 23.1 ± 3.4 kg/m^2, and 31% showed obesity (BMI ≥ 25.0). In subjects without ascites, edema or HCC, mean BMI was 23.6 ± 3.6, and 34% had obesity. Protein malnutrition defined as serum albumin of less than 3.5 g/dL and energy malnutrition as respiratory quotient of less than 0.85 appeared in 61% and 43%, respectively, and protein-energy malnutrition (PEM) in 27% of all subjects. Among subjects without HCC, each proportion was 67%, 48% and 30%, respectively. QOL was significantly lower on all subscales than Japanese national standard values, but was similar regardless the presence or absence of HCC.

Conclusion: While PEM is still present in liver cirrhosis, an equal proportion has obesity in recent patients. Thus, in addition to guidelines for PEM, establishment of nutrition and exercise guidelines seems essential for obese patients with liver cirrhosis.

Key words: body mass index, energy malnutrition, liver cirrhosis, protein malnutrition, quality of life

INTRODUCTION

BECAUSE THE LIVER plays the central role in nutrient and fuel metabolism, protein-energy malnutrition (PEM) is common in patients with liver cirrhosis.[1,2] Moreover, such malnutrition leads to poor prognosis and decline in the quality of life (QOL) of cirrhotics.[3,4]

Branched-chain amino acid (BCAA) administration for protein malnutrition raises the serum albumin level and improves the QOL and survival of patients with liver cirrhosis.[5–8] Treatment with late-evening snack (LES) for energy malnutrition improves respiratory quotient (RQ), liver dysfunction and QOL.[9,10]

Therefore, the guidelines for the treatment of liver cirrhosis by Japanese Society of Gastroenterology,[11] American Society for Parenteral and Enteral Nutrition[12] and European Society for Clinical Nutrition and Metabolism[13] recommend such nutritional therapy.

However, these evidences were obtained in the cirrhotic patients recruited from 1995–2000, where protein or energy malnutrition prevailed in 50–87%.[1–4] In contrast, in the next 10 years, obesity rate in the cirrhotic patients rose to approximately 30%.[14] More recently, non-alcoholic steatohepatitis (NASH) or the

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hepatic inflammation, fibrosis and carcinogenesis due to obesity became the topics.[14–16]

Therefore, it is essential to re-evaluate a nourishment state of the current cirrhotic patients to update the guidelines. In this report, we investigated comprehensive data on the nourishment state and QOL in a large group of patients with liver cirrhosis recruited in the years 2007–2011.

METHODS

Patients

TWO HUNDRED AND ninety-four patients with liver cirrhosis (171 men and 123 women; mean age, 68 ± 10 years) undergoing treatment between 2007 and 2011 were recruited by a Research Group (Gifu University, Hyogo College of Medicine, Aichi Medical University and Saga University) supported by the Ministry of Health, Labor and Welfare of Japan. Liver cirrhosis was diagnosed by clinical and laboratory profiles and by histological examination of liver biopsy specimens. The etiology of cirrhosis was hepatitis B virus in 35 patients, hepatitis C virus in 204, alcohol in 25, NASH in six and others in 24. Child–Pugh classification of the disease severity[17] was A in 154 cases, B in 91 cases and C in 49 cases. One hundred and fifty-eight patients had hepatocellular carcinoma (HCC), and their clinical stage was I in 41 patients, II in 41, III in 54 and IV in 22. Clinical profiles of the patients are presented in Table 1. The proportion of patients supplemented with BCAA or LES rose in parallel with the increasing grade of Child–Pugh classification. Patients with fever, HIV infection, overt infectious disease (septicemia, pneumonia, urinary tract infection), renal insufficiency or under immunomodulatory therapy were excluded. The study protocol was approved by the Medical Ethics Committee of Gifu University Graduate School of Medicine, and informed consent was obtained from all patients. The study protocol was in agreement with the 1975 Declaration of Helsinki as revised in 1983.

Hematological examinations

Blood was drawn for routine laboratory examinations in the early morning after overnight fasting on the day of metabolic studies. Serum albumin, total bilirubin, alt alanine aminotransferase, prothrombin activity and urinary nitrogen (UN) were measured with a standard clinical analyzer at the central laboratory in each hospital.

Nutritional assessment

Metabolic studies were carried out using an indirect calorimeter (Aeromonitor AE-300S; Minato Medical Science, Osaka, Japan) to estimate non-protein re-

Table 1 Clinical and biochemical profiles of patients with liver cirrhosis

	Cirrhosis ($n = 294$)	Child A ($n = 154$)	Child B ($n = 91$)	Child C ($n = 49$)	*P*
Age (years)	68 ± 10	68 ± 10	68 ± 10	68 ± 12	n.s.
Sex (male/female)	171/123	90/64	51/40	30/19	n.s.
Height (cm)	159 ± 9.1	159 ± 9.0	159 ± 9.1	159 ± 9.7	n.s.
Weight (kg)	59 ± 11	58 ± 9.6	59 ± 11	60 ± 13	n.s.
Body mass index (kg/m^2)	23.1 ± 3.4	22.9 ± 3.0	23.4 ± 3.6	23.6 ± 4.0	n.s.
Etiology (HBV/HCV/alcohol/others)	35/204/25/30	20/108/11/15	11/62/8/10	4/34/6/5	n.s.
Hepatocellular carcinoma (+/–)*	158/136	84/69	54/38	20/29	n.s.
Number of patients					
Treated with BCAA	97	35	45	17	<0.01
Supplied with LES	36	8	19	9	<0.01
Albumin (g/dL)	3.3 ± 0.6	3.6 ± 0.5	3.0 ± 0.4	2.6 ± 0.4	<0.01
Total bilirubin (mg/dL)	1.4 ± 1.8	0.9 ± 0.4	1.5 ± 1.2	3.2 ± 3.8	<0.01
Alanine aminotransferase (IU/L)	44 ± 31	43 ± 30	44 ± 29	45 ± 40	n.s.
Prothrombin time (%)	81 ± 30	91 ± 32	75 ± 23	66 ± 22	<0.01

HBV, hepatitis B virus; HCV, hepatitis C virus; BCAA, branched-chain amino acids; LES, late-evening snack; n.s., not significant.
Data are presented as number of patients or mean ± standard deviation.
Statistical analysis was performed by one-way ANOVA or contingency table analysis for distribution among Child–Pugh grades A, B and C.
*Clinical stage of hepatocellular carcinoma was I in 41 patients, II in 41, III in 54 and IV in 22.

spiratory quotient (npRQ) from measured oxygen consumption/min (VO_2), carbon dioxide production/min (VCO_2) and total urinary nitrogen using the following equation:[18–20]

$$npRQ = (1.44Vco_2 - 4.890UN)/(1.44Vo_2 - 6.04UN).$$

Measurements were performed between 07.00 and 09.00 hours while the patients were still lying in bed. The last meal was served at 18.00 hours on the previous day.

We measured height and bodyweight, and calculated body mass index (BMI).

QOL questionnaire

Health-related QOL was measured using the Short Form-8 (SF-8) questionnaire.[21–23] The SF-8 contains eight questions that provide a quantitative evaluation on each of eight subscales: (i) physical functioning (PF); (ii) role physical (RP); (iii) bodily pain (BP); (iv) general health perception (GH); (v) vitality (VT); (vi) social functioning (SF); (vii) role emotional (RE); and (viii) mental health (MH).

Statistical analysis

Data were expressed as the mean and standard deviation. Comparisons of measured values among Child–Pugh classification grade A, B and C were performed using one-way ANOVA. Comparisons of sex, etiology and the presence of HCC among Child–Pugh classification grades were performed using contingency table analysis. Measured QOL was analyzed by *z*-test or Student's *t*-test between each group. Data analysis was performed using JMP ver. 5.1J (SAS Institute Japan, Tokyo, Japan) and $P < 0.05$ was considered statistically significant.

RESULTS

BMI of the patients with liver cirrhosis

THE MEAN BMI of all patients with liver cirrhosis was 23.1 ± 3.4 kg/m^2.

The ratio of obese subjects with BMI of 25 or higher was 30.6% and that of less than 18.5 kg/m^2 was 5.1%, respectively (Fig. 1).

We then excluded patients with ascites, edema or HCC to match the present cohort with those reported in 2002.[4] The number of patents in this cohort was 95, and Child–Pugh grades A, B and C were 71:22:2, respectively. Mean BMI was 23.6 ± 3.6 kg/m^2, and BMI of less than 18.5 kg/m^2 and 25.0 kg/m^2 or higher were observed in 9.2% and 33.7%, respectively (Fig. 2).

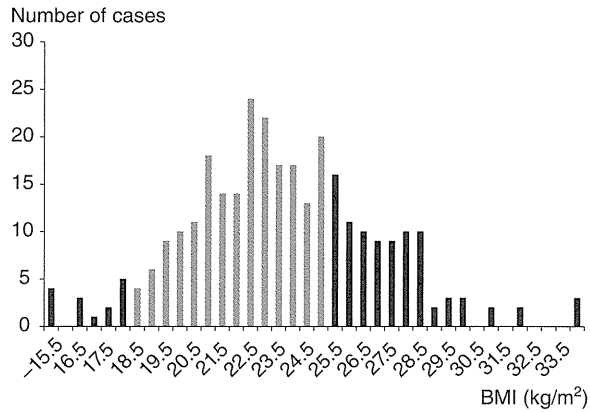

Figure 1 Distribution of body mass index (BMI) in patients with liver cirrhosis. Total number of patients = 294. Obese subjects (BMI ≥ 25) were present in 30.6%, lean ones (18.5 ≤ BMI < 25) were in 64.3% and emaciation (BMI < 18.5) was observed in 5.1%.

Incidence of protein malnutrition, energy malnutrition and PEM in patients with liver cirrhosis

We examined nutritional status in 181 patients with liver cirrhosis that underwent indirect calorimetry. In these patients, the male : female ratio was 112:69, HCC

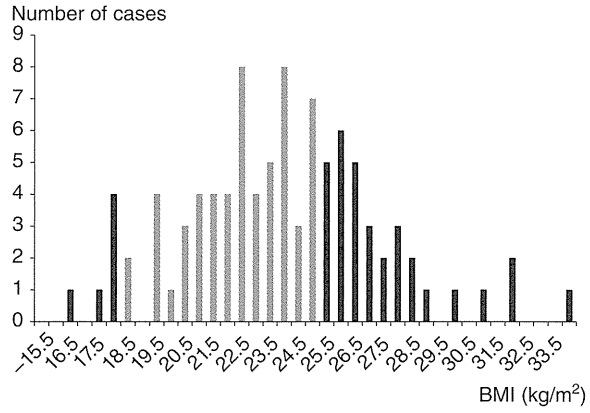

Figure 2 Distribution of body mass index (BMI) in cirrhotic patients without ascites, edema or hepatocellular carcinoma. Total number of patients = 95. Obese subjects (BMI ≥ 25) were present in 33.7%, lean ones (18.5 ≤ BMI < 25) were in 57.1% and emaciation (BMI < 18.5) was observed in 9.2%.

Table 2 Incidence of protein and energy malnutrition in patients with liver cirrhosis

Energy nutritional state	Protein nutritional state	
	Normal (%)	Malnourished (%)
Normal (%)	42 (23%)	62 (34%)
Malnourished (%)	28 (16%)	49 (27%)

Protein malnutrition was defined as serum albumin level of <3.5 g/dL and energy malnutrition as a respiratory quotient of <0.85.
Total number of patients = 181.
Data are presented as number of patients (%).

was present in 94, and Child–Pugh grades A : B : C were 90:58:33. When protein malnutrition was defined as serum albumin level of less than 3.5 g/dL and energy malnutrition as a non-protein respiratory quotient of less than 0.85, protein malnutrition was found in 61%, energy malnutrition in 43% and PEM in 27% (Table 2). Similarly, among 87 patients without HCC (Child–Pugh grades A : B : C, 36:27:24), 67% had protein malnutrition, 48% had energy malnutrition and 30% had PEM (Table 3).

Table 3 Incidence of protein and energy malnutrition in cirrhotic patients without hepatocellular carcinoma

Energy nutritional state	Protein nutritional state	
	Normal (%)	Malnourished (%)
Normal (%)	13 (15%)	32 (37%)
Malnourished (%)	16 (18%)	26 (30%)

Protein malnutrition was defined as serum albumin level of <3.5 g/dL and energy malnutrition as a respiratory quotient of <0.85.
Total number of patients = 87.
Data are presented as number of patients (%).

Table 5 Comparison of health-related quality of life in cirrhotics by the presence or absence of hepatocellular carcinoma

Subscales	Absence of hepatocellular carcinoma	Presence of hepatocellular carcinoma	*P*
Physical functioning	43.4 ± 4.9	44.2 ± 5.5	n.s.
Role physical	41.1 ± 6.3	42.1 ± 6.8	n.s.
Bodily pain	47.8 ± 5.3	48.7 ± 5.1	n.s.
General health perception	44.9 ± 4.5	45.4 ± 3.9	n.s.
Vitality	46.5 ± 4.3	48.4 ± 4.2	n.s.
Social functioning	45.3 ± 5.0	46.8 ± 5.4	n.s.
Role emotional	45.3 ± 5.0	45.8 ± 6.1	n.s.
Mental health	46.6 ± 3.9	48.5 ± 4.0	n.s.

n.s., not significant.
Data are presented as mean ± standard deviation.
Statistical analysis was performed by *z*-test between the presence and absence of hepatocellular carcinoma.

Health-related QOL of the patients with liver cirrhosis

We examined health-related QOL in 114 patients with liver cirrhosis (64 men and 50 women) using the SF-8. Sixty-two patients had HCC, and Child–Pugh grades A : B : C were 63:26:25.

Quality of life of all subjects was significantly lower on all subscales than Japanese national standard values (Table 4),[24] but no difference was observed between the presence and the absence of HCC (Table 5).

Table 4 Comparison of health-related quality of life between the Japanese national standard and the patients with liver cirrhosis

Subscales	Japanese national standard	Patients with liver cirrhosis	*P*
Physical functioning	50.1 ± 5.0	43.8 ± 5.2	<0.01
Role physical	50.2 ± 5.3	41.6 ± 6.6	<0.01
Bodily pain	51.3 ± 8.3	48.3 ± 5.3	<0.01
General health perception	50.6 ± 6.6	45.2 ± 4.4	<0.01
Vitality	52.4 ± 5.5	47.5 ± 4.3	<0.01
Social functioning	50.2 ± 6.6	46.1 ± 5.3	<0.01
Role emotional	51.3 ± 4.5	45.6 ± 5.7	<0.01
Mental health	53.3 ± 5.4	47.6 ± 4.0	<0.01

Data are presented as mean ± standard deviation.
Statistical analysis was performed by Student's *t*-test between the Japanese national standard[24] and the patients with liver cirrhosis.

DISCUSSION

PROTEIN-ENERGY MALNUTRITION is a common manifestation in cirrhotic patients with reported incidences as high as 50–87%.[1,2] Protein nutrition is usually evaluated by serum albumin level and, for energy nutrition, indirect calorimetry is recommended for precise analysis.[13] Energy malnutrition typically shows reduced carbohydrate oxidation, increased fat oxidation and decline in npRQ measured by indirect calorimetry. It is reported that PEM worsens prognosis and QOL in patients with liver cirrhosis.[3,4] Thus, intervention for PEM is an important issue in the clinical management of liver cirrhosis.

For this purpose, BCAA administration for protein malnutrition raises the serum albumin level and improves QOL and survival of patients with liver cirrhosis.[5–8] LES for energy malnutrition improves npRQ, liver dysfunction and QOL.[9,10] Thus, many guidelines[11–13] recommend such nutritional therapy for liver cirrhosis.

However, these evidences were obtained in the cirrhotic patients recruited from 1995 through 2000 where malnutrition prevailed but obesity was apparently less (20%)[4] than the general cohort (30%).[25] In the next 10 years, obesity rose by approximately 1.5 times in the patients with chronic liver disease in Japan.[14] In addition, presence of diabetes mellitus, hyperinsulinemia or obesity is currently regarded as a significant risk factor for liver carcinogenesis.[14–16] Furthermore, the relationship between obesity and liver inflammation and fibrosis, including NASH has become an important issue in recent years. Therefore, it is necessary to elucidate the nourishment state of the present cirrhotic patients to update guidelines. Thus, we report in this paper a comprehensive survey of the nourishment state and QOL in the present patients with liver cirrhosis.

The etiology of the 294 cirrhotics was hepatitis B virus in 11.9%, hepatitis C virus in 69.4%, alcohol in 8.5%, NASH in 2.0% and others in 8.2% in this study. In the 44th Annual Meeting of Japan Society of Hepatology in 2008 (Matsuyama), the reported etiology of 33 379 cirrhotics was hepatitis B virus in 13.9%, hepatitis C virus in 60.9%, alcohol in 13.6%, NASH in 2.1% and others in 9.5%,[26] indicating similar patient composition between two studies.

Obesity is defined by BMI of 25 or higher in Japan but by 30 or higher by World Health Organization. In this study, the mean BMI excluding patients with ascites, edema or HCC was 23.6 ± 3.6 kg/m^2 and the ratio of obese subjects with BMI of 25 or higher was 33.7% of these patients (Fig. 2). The proportion of obese people in the general population of Japan at matched age was 30.5% in 2009.[25] Thus, an equal or greater proportion of patients with liver cirrhosis has obesity than the general population of Japan at present.

The increase in obesity, or excess energy nutrition status, and subsequent impaired glucose metabolism potentially bring about an unfavorable outcome in cirrhotic patients. Actually, excess energy nutrition contributed to induce carcinogenesis in liver cirrhosis,[15,27,28] and the number of obese subjects doubled in the candidates for liver transplantation in the previous 10 years in the USA.[29–31]

As to PEM exactly defined by serum albumin and npRQ, Tajika *et al.* reported that protein malnutrition was identified in 75%, energy malnutrition in 62% and PEM in 50% of 109 patients with liver cirrhosis in 1995.[4] In our study, 87 patients without HCC composed a group to show comparable backgrounds to those by Tajika *et al.*[4] Among them, 67% had protein malnutrition, 48% had energy malnutrition and 30% had PEM (Table 3). Taken together, the protein malnutrition remains almost similar in liver cirrhosis, but the patients with energy malnutrition, particularly PEM, substantially decreased.

The above-mentioned results urge that two concerns are addressed. The first is the effect of altered nutritional state of cirrhotics on their QOL, and the second is a question if exercise should be prescribed for obese cirrhotics. Regarding QOL, reduction in bodyweight achieved by chronic liver disease patients with obesity was associated with improved liver dysfunction, histology or QOL.[32,33]

In this study, basal QOL was estimated by the SF-8, and was significantly lower on all subscales than Japanese national standard values. However, no difference was observed by the presence or absence of HCC. In contrast, QOL of cirrhotic patients significantly correlated with the grade of disease severity as defined by the Child–Pugh classification (data not shown). It was thus suggested that the degree of the hepatic functional reserve contributed to a greater extent than the progression of cancer as for QOL of cirrhotic patients.

In conclusion, while PEM is still present in liver cirrhosis, a greater proportion shows obesity in Japanese patients at present. Because exacerbated inflammation, fibrosis and carcinogenesis has been reported in obese patients with liver cirrhosis, the present findings urge revision of nutritional and, possibly, establishment of exercise guidelines for obese patients with liver cirrhosis, in addition to the current PEM guidelines.