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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 InStat 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

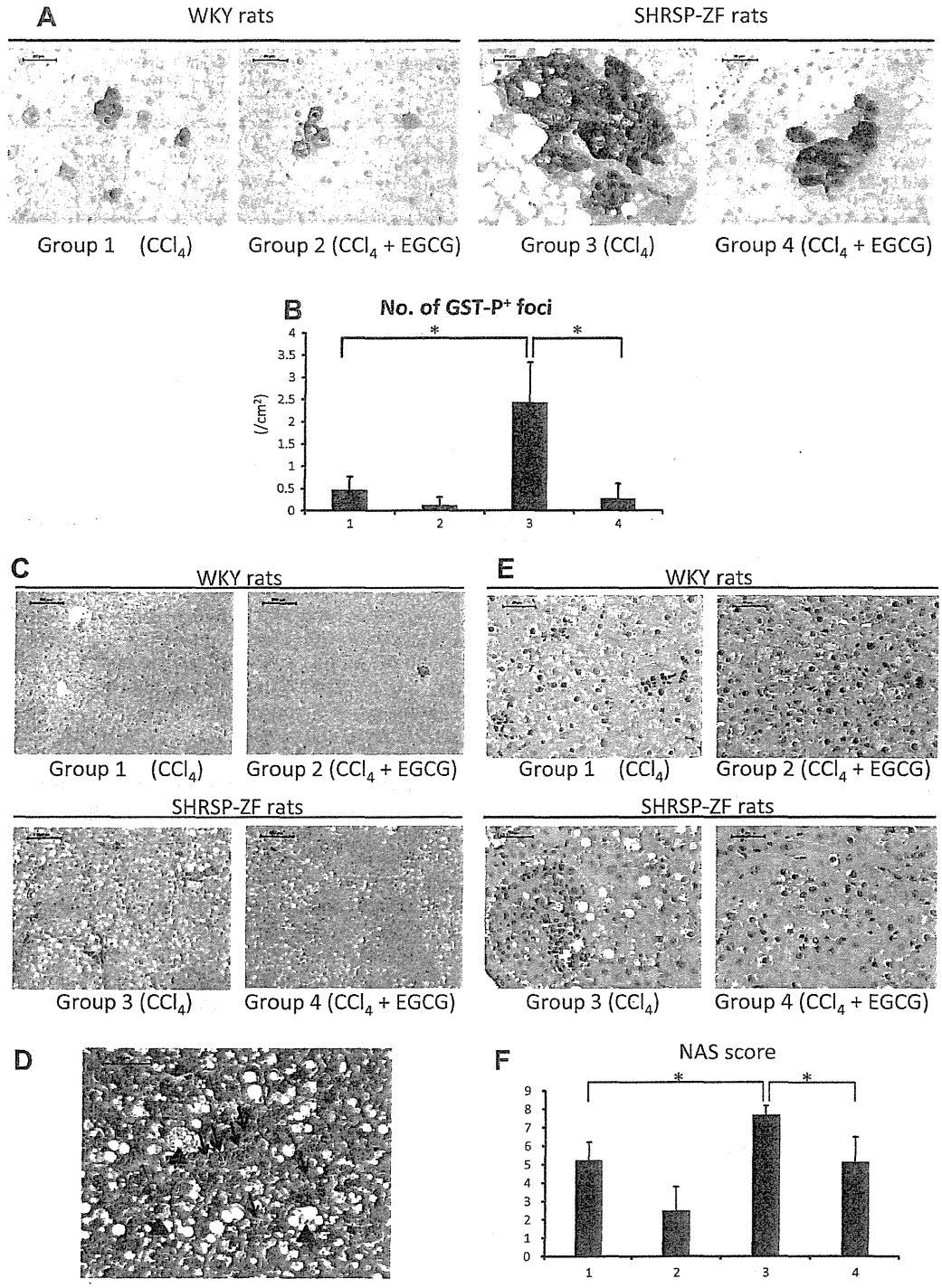

Fig. 1. Effects of EGCG on the development of GST-P$^+$ foci and histopathology in the livers of the experimental rats. (A) Representative photomicrographs of GST-P$^+$ foci and (B) the average number of GST-P$^+$ foci that developed in the livers of the experimental rats. Group 1: WKY rats treated without 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.

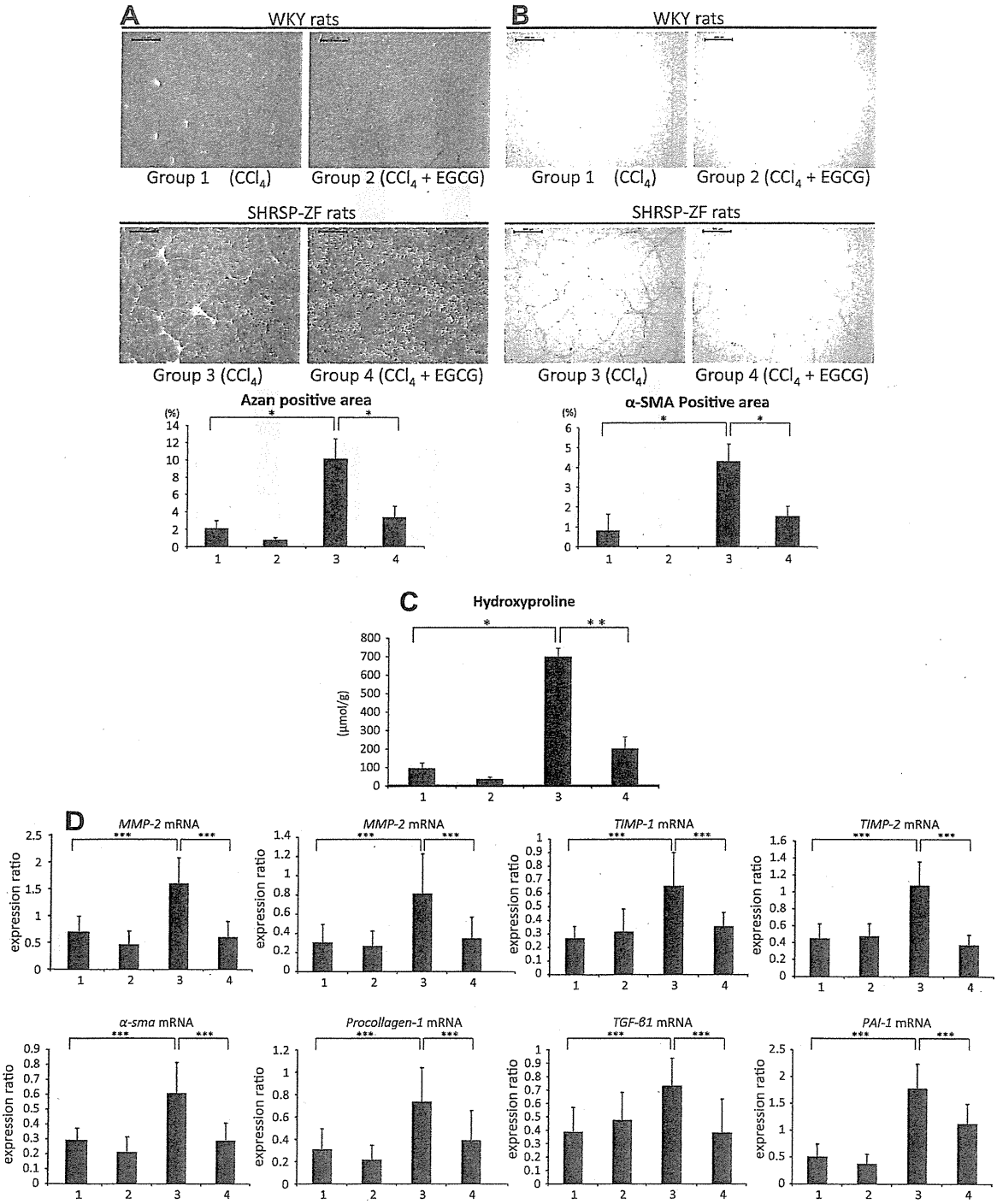

Fig. 2. Effects of EGCG on hepatic fibrosis in the experimental rats. (A) Representative photomicrographs of liver sections stained with Azan stain to show fibrosis (upper panels). The hepatic fibrosis area was evaluated by Azan stain (lower panel). (B) Immunohistochemical detection of α-SMA expression 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; $P < 0.05$).

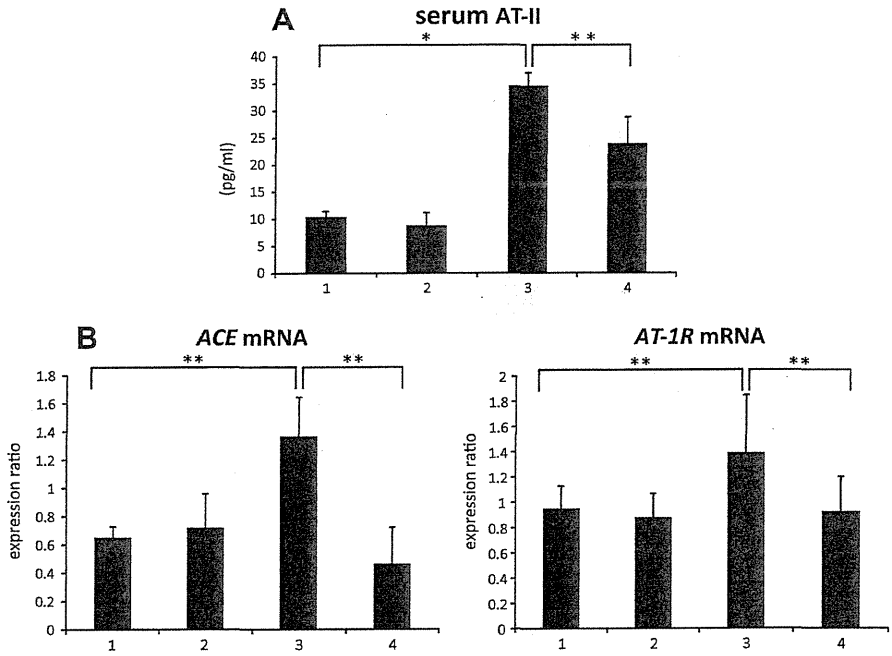

Fig. 3. Effects of EGCG on renin-angiotensin system in the experimental rats. (A) The serum concentrations of AT-II were measured using enzyme immunoassay. (B) The expression levels of ACE and AT-1R mRNA in the livers of the experimental rats were examined by quantitative real-time RT-PCR by using specific 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,

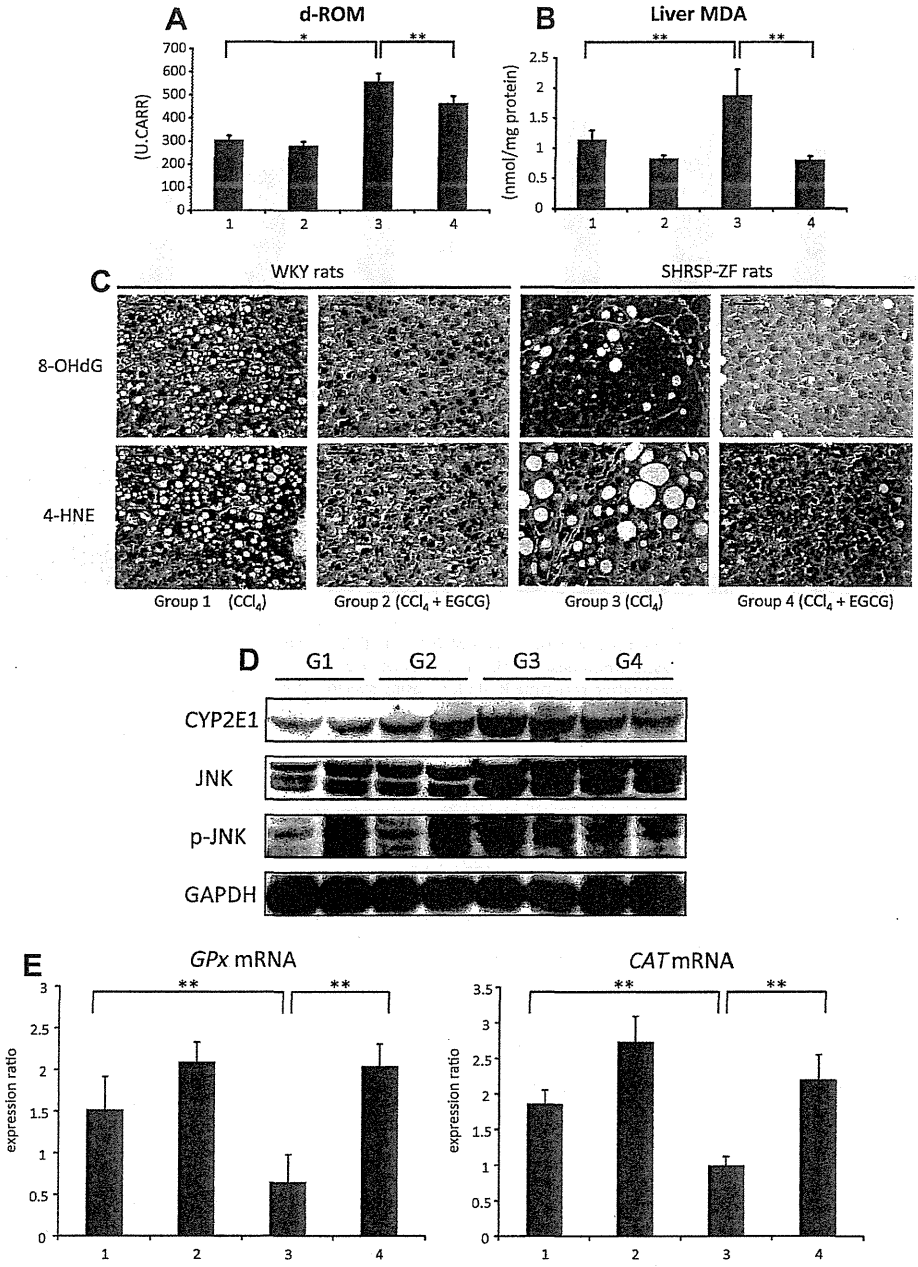

Fig. 4. Effects of EGCG on the serum levels of d-ROM, hepatic concentration of MDA, hepatic expression levels of 8-OHdG, 4-HNE, CYP2E1, JNK, and p-JNK proteins, and hepatic expression levels of GPx and CAT mRNA in the experimental rats. (A) Hydroperoxide levels in the serum were determined by the d-ROM 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-

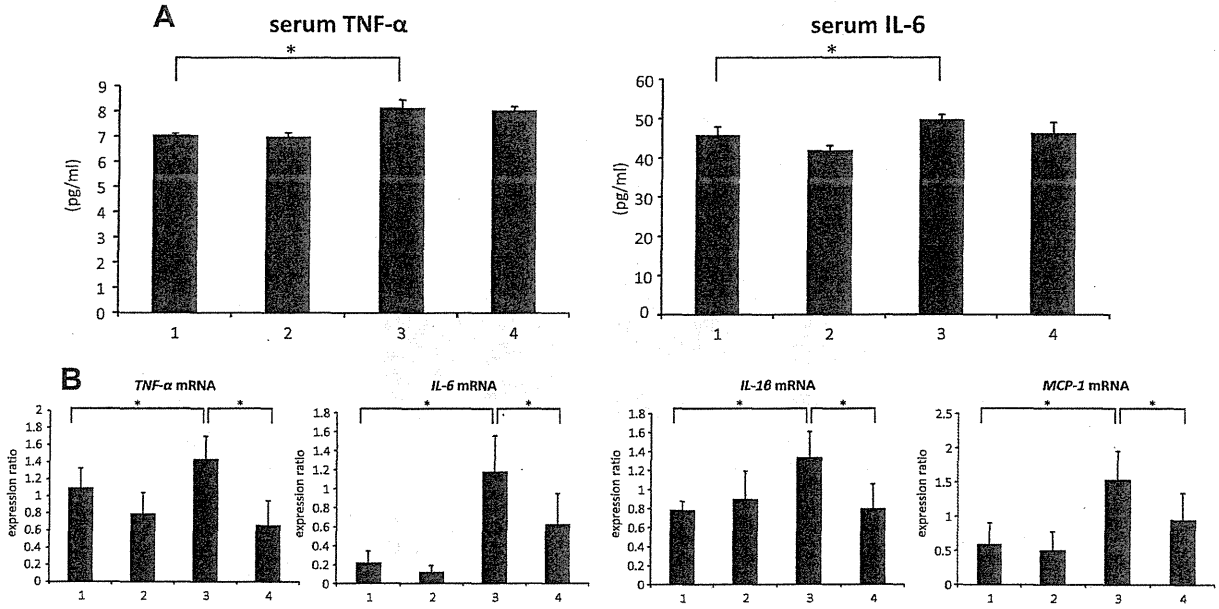

Fig. 5. Effects of EGCG on the serum levels of TNF-α and IL-6 and the expression levels of TNF-α, IL-6, IL-1β, and MCP-1 mRNA in the livers of the experimental rats. (A) The serum concentrations of TNF-α and IL-6 were measured by enzyme immunoassay. (B) Total RNA was isolated from the livers of experimental 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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REVIEW

Pharmaceutical and nutraceutical approaches for preventing liver carcinogenesis: Chemoprevention of hepatocellular carcinoma using acyclic retinoid and branched-chain amino acids

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The poor prognosis for patients with hepatocellular carcinoma (HCC) is associated with its high rate of recurrence in the cirrhotic liver. Therefore, more effective strategies need to be urgently developed for the chemoprevention of this malignancy. The malfunction of retinoid X receptor α, a retinoid receptor, due to phosphorylation by Ras/mitogen-activated protein kinase is closely associated with liver carcinogenesis and may be a promising target for HCC chemoprevention. Acyclic retinoid (ACR), a synthetic retinoid, can prevent HCC development by inhibiting retinoid X receptor α phosphorylation and improve the prognosis for this malignancy. Supplementation with branched-chain amino acids (BCAA), which are used to improve protein malnutrition in patients with liver cirrhosis, can also reduce the risk of HCC in obese cirrhotic patients. In experimental studies, both ACR and BCAA exert suppressive effects on HCC development and the growth of HCC cells. In particular, combined treatment with ACR and BCAA cooperatively inhibits the growth of HCC cells. Furthermore, ACR and BCAA inhibit liver tumorigenesis associated with obesity and diabetes, both of which are critical risk factors for HCC development. These findings suggest that pharmaceutical and nutraceutical approaches using ACR and BCAA may be promising strategies for preventing HCC and improving the prognosis of this malignancy.

Keywords:
Acyclic retinoid / Branched-chain amino acid / Chemoprevention / Hepatocellular carcinoma / Obesity

1 Introduction

Hepatocellular carcinoma (HCC), which usually develops in the livers of patients with chronic hepatitis and liver cirrhosis, is a serious clinical and social issue worldwide. Annually, the number of new cases is approximately 750 000, with an estimated 700 000 patients dying because of the malignancy [1, 2]. Although effective methods of diagnosis and treatment for HCC have been recently developed, improvement in the prognosis for this cancer is limited; overall survival, 10 years after curative treatment, is only 22–35% [3, 4]. The primary reason for the poor prognosis of HCC is its high frequency of recurrence after curative treatment; the recurrence rate, 5 years after definitive therapy in cirrhotic patients, may exceed 70% [5–7]. These facts indicate that curative treatment for HCC is difficult once this malignancy has developed, and therefore, effective strategies for preventing this cancer are urgently required.

In a previous, prospective, randomized trial, we reported that the oral administration of acyclic retinoid (ACR), a novel synthetic retinoid, significantly suppressed the posttherapeutic recurrence of HCC and improved the survival rate of patients [8–10]. Oral supplementation with branched-chain

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Abbreviations: **ACR**, acyclic retinoid; **BCAA**, branched-chain amino acids; **ERK**, extracellular signal-regulated kinase; **HBV**, hepatitis B virus; **HCC**, hepatocellular carcinoma; **HCV**, hepatitis C virus; **IGF**, insulin-like growth factor; **IGF-1R**, IGF-1 receptor; **MAPK**, mitogen-activated protein kinase; **PEM**, protein energy malnutrition; **PI3K**, phosphoinositide-3-kinase; **RAR**, retinoic acid receptor; **RTK**, receptor tyrosine kinase; **RXR**, retinoid X receptor

amino acids (BCAA), which is widely used in patients with liver cirrhosis to improve protein energy malnutrition (PEM), also reduced the risk of HCC in obese cirrhotic patients [11]. The effects of ACR and BCAA on the chemoprevention of HCC and the inhibition of HCC cell growth have been reported in several experimental studies [12–16]. In particular, recent rodent studies demonstrated that administration of ACR and BCAA suppresses the liver carcinogenesis associated with obesity and diabetes, both of which are critical risk factors for HCC development [17, 18]. The results of these clinical and basic studies strongly suggest that pharmaceutical and nutraceutical approaches, especially using ACR and BCAA, might be effective strategies for preventing liver carcinogenesis. In this article, we provide an overview of the clinical characteristics and molecular pathogenesis of HCC, focusing on the role of retinoid X receptor α (RXRα) phosphorylation in liver carcinogenesis. The detailed effects of ACR and BCAA in the prevention of HCC development are reviewed, based on our clinical and basic research. We also review the possibility of pharmaceutical and nutraceutical approaches for the inhibition of obesity- and diabetes-related liver carcinogenesis through the targeting of the pathophysiological conditions caused by these metabolic abnormalities, concentrating on the effects of ACR and BCAA.

2 Clinical characteristics of HCC

Most cases of HCC, which is the dominant form of primary liver carcinoma, are associated with the chronic inflammation and subsequent cirrhosis of the liver, that is induced by a persistent infection with one of the hepatitis viruses, hepatitis B virus (HBV) or hepatitis C virus (HCV) [19, 20]. After development of virus-induced chronic hepatitis and liver cirrhosis, the entire liver enters a precancerous state, possessing multiple, independent, premalignant, or latent malignant clones. Therefore, the typical clinical pattern of liver carcinogenesis is **ulticentric carcinogenesis, which is also described as **ield cancerization. This carcinogenesis pattern contributes to the high frequency of HCC development in patients with viral liver cirrhosis. Significantly, the annual rate for HCC development is approximately 7% in cirrhotic patients, and even after curative treatment, the annual incidence of recurrence is approximately 20–25% [5–7]. These facts highlight the poor prognosis of viral liver cirrhotic patients and suggest the possibility of improved clinical outcomes if effective strategies are developed for preventing HCC.

One of the most effective approaches for preventing the development of HCC is the eradication of the hepatitis viruses. Several meta-analyses have shown the effectiveness of IFN therapy for preventing HCV-related HCC [21–23], indicating that sustained antiviral response to IFN-based therapy is associated with a reduced risk of developing this malignancy. In addition, IFN treatment might be effective for preventing HCC development in HCV patients, even if sustained antiviral response is not achieved [24]. Antiviral treatments, such as IFN therapy and nucleos(t)ide analog therapy, also prevent the development of HBV-related HCC [25, 26]. These clinical evidences strongly suggest that antiviral treatment is effective for reducing the incidence of HCC development in patients with chronic HBV or HCV infections. In addition, two cohort studies of HCV patients demonstrated that hepatic inflammation alleviation therapy, involving glycyrrhizin injection, suppressed HCC development [27, 28]. These results also indicate that attenuation of chronic inflammation might be effective for inhibiting liver carcinogenesis.

3 Molecular pathogenesis of HCC

HCC is a heterogeneous tumor because it develops in a complex multistep process in which many signaling cascades are altered. That is, the accumulation of genetic alterations is critically involved in hepatocarcinogenesis [29, 30]. Genomic mutations in the *p53* tumor suppressor gene occur in 10–35% of HCC cases [31]. Genomic mutations in the *CTNNB1* gene, which encodes β-catenin, have also identified in approximately 20–40% of liver cancers [31]. Because of these alterations, several signaling pathways related to cell proliferation and survival are activated during liver carcinogenesis. For instance, epithelial growth factor receptor, which is a receptor tyrosine kinase (RTK), is expressed in 68% of HCC cases, and this receptor is associated with the proliferation and clinical stage of this malignancy [32]. Activation of insulin-like growth factor (IGF) 1 receptor (IGF-1R) signaling, which is another RTK, also contributes to the early stages of liver carcinogenesis [30]. The major signaling pathways activated by the RTKs/Ras pathways are the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) and the phosphoinositide-3-kinase (PI3K)/Akt pathways, both of which play important roles in the proliferation and survival of cancer cells. These reports, therefore, strongly suggest that targeting specific RTKs and their downstream signaling pathways is a potentially effective strategy for preventing some types of human malignancies, including HCC [33–38].

4 Retinoid abnormalities and HCC

In addition to the pathophysiological mechanisms as mentioned above, recent studies have revealed the magnitude of the abnormalities in the expression and function of retinoids on liver carcinogenesis [12–15, 39]. Retinoids are a group of natural and synthetic molecules that are structurally and/or functionally related to fat-soluble vitamin A. These molecules participate in a broad spectrum of biological activities, including embryogenesis, growth, differentiation, proliferation, apoptosis, and metabolism [40–42]. The fundamental effects of retinoids on cellular activities are largely mediated through the expression of two distinct families of nuclear receptors, the retinoic acid receptors (RARs) and RXRs. The RARs are activated by all-*trans*-retinoic acid and 9-*cis*-retinoic

acid, with similar affinities, whereas RXRs are only activated by 9-*cis*-retinoic acid [40–42]. Both the RARs and RXRs are composed of three subtypes (α, β, and γ), which are characterized by a modular domain structure, and these nuclear receptors are ligand-dependent transcription factors [40–42]. After ligand binding, the RXRs form homodimers and heterodimers with the RARs and interact with the retinoid X response element or the RAR responsive element, which are located in the promoter region of the target genes, thereby modulating gene expression [40–42]. RXRs can also form heterodimers with other nuclear receptors, such as peroxisome proliferator-activated receptor, indicating that RXRs act as common heterodimerization partners for various types of nuclear receptors [41]. Thus, RXRs are considered the master regulators of nuclear receptors because they are involved in the regulation of fundamental cell activities, including normal cell proliferation, metabolism, and death (regulation of apoptosis). In particular, RXRα plays a critical role in the normal control of hepatocyte lifespan and proliferation [43, 44].

These characteristics also suggest that abnormalities in the expression and function of retinoid signaling are closely associated with deviations from normal cell proliferation and death, which are key factors in the development of several types of human cancers, including HCC. For example, retinol, a transport form of retinoid in the plasma, is locally deficient in HCC, but not in the adjacent, normal liver tissue in a rodent model of hepatocarcinogenesis [45]. In a rat model of chemically induced liver carcinogenesis, repression of RXRα occurs even in the early stages of carcinogenesis because its expression is decreased not only in HCC and liver cell adenoma, but also in precancerous HCC lesions [46]. The expression levels of RARβ, which is regarded as a tumor suppressor gene because of its ability to regulate cell growth and apoptosis [47], are markedly decreased in both human [48] and rat HCC [46]. On the other hand, RARγ, which is overexpressed in human HCC tissues and cells, enhances the growth of HCC cells through the activation of the PI3K/Akt signaling pathway [49]. These reports strongly indicate that the restoration of the function and expression of retinoid receptors, via treatment with retinoids, might be effective for the prevention of certain types of human malignancies, including HCC [12–15, 50, 51].

5 RXRα phosphorylation and HCC

We proposed that RXRα phosphorylation and its malfunction is closely associated with liver carcinogenesis [12–15]. RXRα protein, which is anomalously phosphorylated at its serine and threonine residues, prominently accumulates in both surgically resected human HCC tissues and human HCC-derived cell lines [39, 52]. Activation of the RTK/Ras/MAPK signaling frequently occurs in HCC cells [30, 32]. The constitutive phosphorylation of serine-260 in RXRα, a MAPK/ERK consensus site, by this signaling pathway is closely associated with retarded degradation of RXRα, lowered transcriptional activity of this nuclear receptor, and promotion of cancer cell growth [39, 53]. In human HCC cells, phosphorylated RXRα is resistant to proteolytic degradation via the ubiquitination-/proteasome-mediated pathway, facilitating the accumulation of this phosphorylated protein within HCC tissues [54]. Furthermore, phosphorylated RXRα abolishes its ability to form heterodimers with RARβ, and this is implicated in uncontrolled cell growth and retinoid resistance [55]. These findings suggest that the accumulation of phosphorylated RXRα, regarded as the nonfunctional form of RXRα, may interfere with the function of normal (unphosphorylated) RXRα in a dominant-negative manner, thus, playing a critical role in liver carcinogenesis (Fig. 1). On the other hand, the abrogation of RXRα phosphorylation by a MAPK inhibitor or transfection with the nonphosphomimetic mutant RXRα restores the degradation of RXRα in a ligand-dependent manner [39, 53]. Thus, the targeting of RXRα phosphorylation might be a strategy for preventing HCC, and ACR is a promising agent for this purpose, as discussed in Section 6.

6 Mechanisms of ACR in HCC chemoprevention

ACR, also known as NIK-333 and Peretinoin (Kowa Pharmaceutical, Tokyo, Japan), is a synthetic retinoid that was initially developed as an agonist for both RXR and RAR [56, 57]. ACR inhibits growth of human HCC-derived cells by activating the promoter activity of retinoid X response element and RAR responsive element and regulating the expression of retinoid target genes, including *RARβ*, $p21^{CIP1}$, and *cyclin D1*, resulting in the induction of apoptosis and cell cycle arrest in the G_0–G_1 phase [53, 58–63]. These findings indicate that ACR exerts growth inhibitory effects in HCC cells, at least in part, by working as a ligand for retinoid receptors and controlling their target genes, especially *RARβ* and $p21^{CIP1}$. The antitumor effects of ACR are also associated with suppression of telomerase activity, attenuation of oxidative stress, and inhibition of angiogenesis [64–66]. Moreover, the suppressive effects of ACR on liver carcinogenesis have been demonstrated in several animal experiments [17, 45, 67–69].

Furthermore, we have proposed that inhibition of RXRα phosphorylation is a critical mechanism of ACR, allowing it to exert chemopreventive effects in liver carcinogenesis. In human HCC-derived cells, ACR can restore RXRα function by inactivating the Ras/MAPK signaling system and dephosphorylating RXRα, although 9-*cis*-retinoic acid is incapable of suppressing ERK and RXRα phosphorylation [53]. Moreover, recent studies have revealed that ACR suppresses the growth of several types of cancer cells, such as HCC and head and neck squamous cell carcinoma cells, and prevents chemically induced liver carcinogenesis by inhibiting the activation and expression of several types of growth factors and their corresponding RTKs [63, 66, 68–73]. ACR also inhibits Ras activation, and this is associated with prevention of obesity-related liver tumorigenesis in mice and the

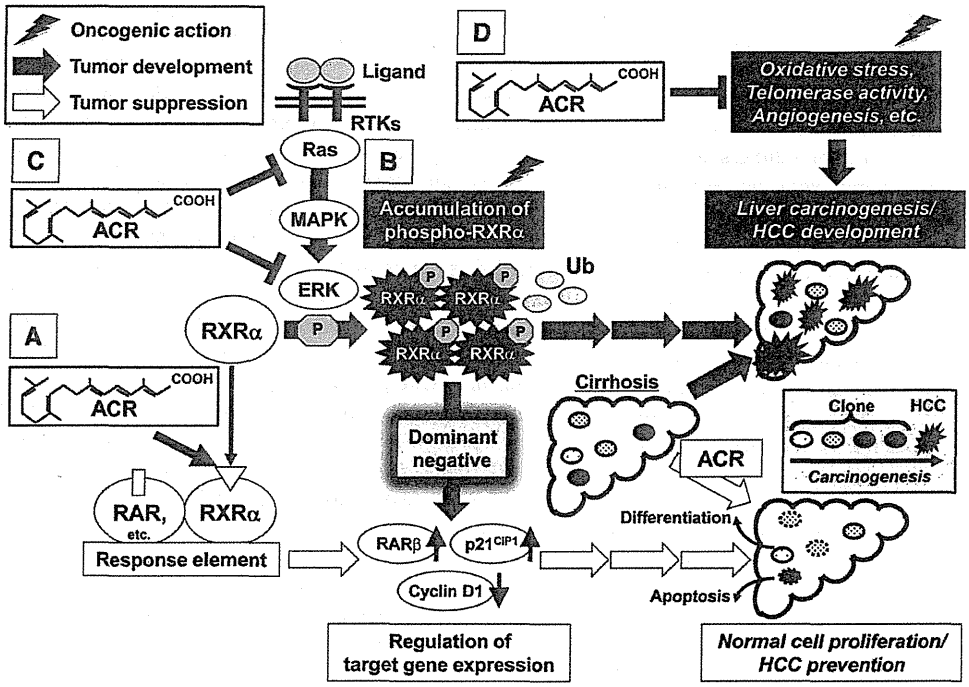

Figure 1. Retinoid refractoriness due to phosphorylation of RXRα, and its restoration by ACR in liver carcinogenesis. When ACR binds to and activates RXRα in normal hepatocytes, the receptor forms homodimers and/or heterodimers with other nuclear receptors, including RARs. This results in the expression of the target genes, such as *RARβ*, *p21^{CIP1}*, and *cyclin D1*, which regulate normal cell proliferation and differentiation and control the induction of apoptosis and cell cycle progression. Therefore, in the cirrhotic liver, ACR can delete and inhibit malignant clones, at least in part, by controlling the expression of these RXRα-target genes (A). In HCC cells, several types of RTKs, such as epidermal growth factor receptor superfamily and IGF-1R and their downstream Ras/MAPK pathway, are highly activated, which results in the phosphorylation of ERK and RXRα and subsequent suppression of dimer formation and transactivation functions of RXRα (refractoriness to retinoid). Furthermore, nonfunctional phosphorylated RXRα, which is sequestered from ubiquitin (Ub)/proteasome-mediated degradation and accumulates in liver cells, interferes with the physiological functions of the remaining nonphosphorylated (i.e., functional) RXRα in a dominant-negative manner, and this is also involved in liver carcinogenesis (B). ACR inhibits phosphorylation of RXRα, restores the function of this receptor, and activates the transcriptional activity of the responsive element associated with this receptor. This is accomplished by inhibiting the Ras/MAPK signaling pathway and the ligand-dependent (growth factor) RTK activities, which contribute to the prevention of liver carcinogenesis and suppression of growth in HCC cells (C). In addition, ACR inhibits growth of HCC cells through the attenuation of oxidative stress, inhibition of telomerase activity, and repression of angiogenesis (D). The pleiotropic effects of ACR to prevent HCC development have also been summarized in recent reviews [12–15].

inhibition of cell growth in human HCC and pancreatic cancer cells [17, 58, 74]. These findings indicate that activation of the RTK/Ras/MAPK signaling pathway, which is involved in HCC development [30, 32], and the subsequent phosphorylation of RXRα are critical targets of ACR for the inhibition of liver carcinogenesis [12–15] (Fig. 1).

7 HCC chemoprevention by ACR: Clinical trial results

Because the results from numerous preclinical experiments indicated that ACR may be an effective agent for HCC chemoprevention, an early-phase, randomized, controlled clinical trial was conducted to determine whether ACR can reduce the incidence and recurrence of second primary HCC in patients who underwent potentially curative treatment for initial HCC [8–10]. In this trial, oral administration of ACR (44 patients, 600 mg/day) for 12 months significantly reduced the incidence of recurrent or new HCC compared to placebo (45 patients) after a median follow-up period of 38 months; 12 patients (27%) in the ACR group developed HCC as compared with 22 patients (49%) in the placebo group ($p = 0.04$) [8]. After a further follow-up period of 62 months, ACR treatment demonstrated improved recurrence-free survival ($p = 0.002$) and overall survival ($p = 0.04$) [9]. The relative risk for the development of secondary HCC and death were 0.31 (95% confidence interval [CI], 0.12–0.78) and 0.33 (95% CI, 0.11–0.79), respectively [8, 9]. Therefore, the estimated 6-year overall survival was 74% in the ACR group and 46% in the placebo group [9].

A multicenter, large-scale ($n = 401$), randomized, placebo-controlled trial also confirmed the effectiveness of ACR in preventing second primary HCCs in HCV-positive patients

who underwent curative treatment for primary or the first recurrence of HCC, with a median follow-up of 2.5 years. In this trial, oral administration of ACR (600 mg/day) had a strong effect on the prevention of a second primary HCC with a hazard ratio of 0.27 (95% CI, 0.07–0.96), 2 years after treatment, and at 3 years, the cumulative recurrence-free survival rates in the ACR-treated group (43.7%) were higher than those in the placebo group (29.3%) [75]. In addition, a subgroup analysis of this study showed that ACR prevented development of a second primary HCC with a hazard ratio of 0.38 (95% CI, 0.20–0.71) in patients who were Child-Pugh A and had small tumors (size, <20 mm) [76]. These results indicated that ACR administration at an early stage of liver cirrhosis contributes to the prevention of HCC. In addition to the effectiveness of ACR for the prevention of HCC development, the results of these clinical trials [8–10, 75, 76], together with a phase I pharmacokinetics trial [77], have proven the safety of ACR in a clinical setting. Therefore, the findings of these clinical trials [8–10, 75–77] strongly suggest that ACR is a novel first-line therapy for reducing the development of a second primary HCC.

8 HCC chemoprevention by ACR: The concept of "clonal deletion" therapy

Two interesting facts were revealed in an early-phase, ACR clinical trial [8–10]. First, the preventive effects of ACR on HCC development lasted up to 50 months after randomization or 38 months after completion of ACR administration, indicating that a 12-month administration of this agent conferred a long-term effect on the prevention of second primary HCCs [10]. Second, ACR administration for 12 months significantly reduced the serum levels of lectin-reactive α-fetoprotein factor 3, which might be produced from latent (i.e., invisible) malignant clones in the remnant liver [78]. These facts suggest the following two possibilities: (i) ACR can delete the α-fetoprotein factor 3 producing premalignant clones from the remnant liver before they expand into clinically detectable HCC and (ii) after the elimination of the malignant clones from the remnant liver by ACR, several years elapse before the clinical appearance of the next HCC clones. The cirrhotic liver is a precancerous field that possesses multiple, independent premalignant, or latent malignant clones. Therefore, before expanding into clinically detectable tumors, a positive approach for the removal and inhibition of such latent malignant clones from the cirrhotic liver should be conducted to prevent HCC development. We consider that implementation of this approach, termed clonal deletion therapy, is a practical approach for preventing HCC, and that ACR is a consistent and reasonable agent for this purpose [12–15] (Fig. 1).

A recent study by Honda et al. [79] reported that an 8-wk administration of ACR significantly elevated the expression levels of many retinoid target genes and tumor suppressor-related genes, but decreased the expression levels of tumor progression-related genes in the liver of HCV-positive patients. This report may also provide evidence that ACR can change the hepatic environment to a non-hypercarcinogenic one.

9 BCAA supplementation and chronic liver disease

BCAA (valine, leucine, and isoleucine) is a widely accepted therapy for improving hepatic insufficiency and its related PEM, which is a common manifestation of patients with liver cirrhosis [80, 81]. PEM affects the outcome of the cirrhotic patients by determining both their quality of life and survival [82, 83]. Cirrhotic patients frequently demonstrate a decreased serum ratio of BCAA to aromatic amino acids, reduced serum albumin levels, and decreased skeletal muscle volume [80, 81]. They have also demonstrated that an increased consumption of foods containing high BCAA content does not affect plasma BCAA levels [84]. On the other hand, nutritional intervention with BCAA has been shown to increase the serum albumin concentration and improve patient quality of life and prognosis by preventing severe complications associated with this disease [85–88]. For instance, in a multicenter, large scale ($n = 646$), randomized, and nutrient intake-controlled trial in Japan, the long-term survival study, oral supplementation with BCAA (12 g/day) for 2 years to patients with decompensated cirrhosis significantly decreased the incidence of events associated with progression to hepatic failure (hazard ratio, 0.67; 95% CI, 0.49–0.93; $p = 0.015$; median observation period, 445 days) [85]. The reports of the trial [85–88], therefore, indicated that BCAA supplementation may serve as a first-line therapy for patients with decompensated cirrhosis.

10 HCC chemoprevention by BCAA supplementation

Several experimental studies have revealed the precise mechanisms of BCAA in the suppression of cancer cell growth and chemoprevention of HCC. Hagiwara et al. [89] reported that BCAA directly suppresses HCC cell proliferation by inducing apoptosis and inhibiting the activation of PI3K/Akt and nuclear factor-κB signaling pathways. BCAA treatment also inhibits the proliferation of human HCC-derived cells by increasing cellular levels of $p21^{CIP1}$ and arresting the cell cycle in the G_0/G_1 phase [90]. Both in vitro and in vivo studies have demonstrated the antiangiogenesis activity of BCAA induced by suppressing the expression of vascular endothelial growth factor in HCC cell lines and in the liver of rats bearing neoplasm [91, 92]. BCAA supplementation also reduces oxidative stress in HCV-positive patients with liver cirrhosis as well as in rats with advanced liver cirrhosis [93, 94]. These reports suggest that BCAA exerts chemopreventive effects against HCC, at least in part, by suppressing angiogenesis and

improving oxidative stress, both of which are critically involved in liver carcinogenesis.

Moreover, recent clinical trials revealed that BCAA supplementation may influence the prevention of HCC development [11, 95–100]. The results of a retrospective analysis showed that BCAA supplementation (12 g/day for >6 months) reduced the incidence of HCC in patients with liver cirrhosis with a hazard ratio of 0.42 (95% CI, 0.22–0.80; $p = 0.009$) [95]. Oral supplementation of BCAA (12 g/day for 6 months) significantly decreased the serum levels of AFP and reduced early recurrence after hepatic resection in patients with HCC [98]. In a subset analysis of the long-term survival study, Muto et al. also showed that long-term oral supplementation with BCAA significantly inhibited the development of HCC in type C cirrhotic patients with BMIs >25 [11]. Moreover, the administration of BCAA granules (12 g/day for 60 months) markedly inhibited the cumulative recurrence of HCC, after curative treatment in patients, with insulin resistance [96]. Therefore, long-term treatment with BCAA is an effective strategy for improving the clinical outcomes in cirrhotic patients by reducing the likelihood of liver failure and in obese and diabetic patients, by suppressing liver carcinogenesis. Pathophysiological conditions involved in the development of obesity-related HCC and in the precise mechanisms of BCAA to inhibit liver carcinogenesis, in particular the mechanisms associated with obesity, are discussed in the following sections.

11 Obesity and HCC

Among patients with liver cirrhosis, the proportion of obese subjects is gradually increasing [101, 102]. This is a serious problem when considering the medical care of chronic liver disease because obesity and its related metabolic abnormalities, especially diabetes mellitus, are major risk factors for the development of HCC [11, 103–106]. Nonalcoholic fatty liver disease, a hepatic manifestation of obesity and metabolic syndrome, is also an important healthcare problem, especially in developed countries, since it can progress to nonalcoholic steatohepatitis), which in turn leads to liver cirrhosis and HCC development [107, 108].

Recent studies have shown several pathophysiological mechanisms linking obesity and liver carcinogenesis, including the emergence of insulin resistance, activation of the IGF/IGF-1R axis, development of a state of chronic inflammation, induction of oxidative stress, and adipokine imbalance [103, 104]. In particular, insulin resistance, which leads to systemic and hepatic inflammation, liver steatosis, and activation of the IGF/IGF-1R axis, is considered to play a critical role in the development of HCC [35, 103, 104, 109]. On the other hand, these reports strongly indicate that targeting such pathophysiological disorders via pharmaceutical and nutraceutical intervention might be an effective strategy to prevent obesity-related liver carcinogenesis [16, 110]. For instance, pitavastatin, a drug widely used for the treatment of hyperlipidemia, and (−)-epigallocatechin-3-gallate, one of the green tea catechins, significantly inhibit the obesity-related liver tumorigenesis by attenuating the chronic inflammation induced by excess fat deposition [111, 112]. Administration of ACR also suppresses diethylnitrosamine-induced liver tumorigenesis in obese and diabetic mice and this is associated with inhibition of Ras activation and phosphorylation of the ERK and RXRα proteins [17]. Increase in insulin sensitivity and the attenuation of systemic and hepatic inflammation by ACR also contribute to this inhibition [17], indicating that ACR might be useful in the chemoprevention of obesity-related HCC (Fig. 2).

12 Preventive mechanisms of BCAA in obesity-related liver carcinogenesis

Recent experimental studies have revealed that BCAA improves insulin resistance and glucose tolerance via the enhancement of glucose metabolism in skeletal muscle, adipose tissue, and the liver [113–118]. Improvements in insulin resistance and glucose tolerance, by oral BCAA supplementation in chronic liver disease patients, have also been reported in several clinical trials [119–121]. In addition, a recent in vitro study showed that BCAA treatment suppresses insulin-induced proliferation of HCC cells by inhibiting the insulin-induced activation of the PI3K/Akt pathway and the subsequent antiapoptotic pathway [89]. We, therefore, consider that improvements in glucose metabolism and insulin resistance might be a critical mechanism in the reduction of the incidence of HCC development in obese cirrhotic patients [11]. This hypothesis was evaluated using an obesity- and diabetes-related liver carcinogenesis mouse model [18]. In the model, BCAA supplementation significantly inhibited diethylnitrosamine-induced liver tumorigenesis in obese and diabetic *db/db* mice by improving liver steatosis and fibrosis, insulin resistance, and hyperleptinemia [18]. Supplementation with BCAA also inhibited the spontaneous development of hepatic premalignant lesions in *db/db* mice via the attenuation of chronic inflammation in both the liver and white adipose tissue [122]. Moreover, BCAA treatment significantly inhibited the proliferation of human HCC-derived cells induced by visfatin, a serum adipokine that is significantly correlated with stage progression and tumor enlargement of HCC [90]. Yoshiji et al. [92] also reported that, in obese and diabetic rats exhibiting insulin resistance, BCAA treatment significantly exerted a chemopreventive effect against HCC through the suppression of hepatic neovascularization. The results of these reports [18, 89, 90, 92, 122] strongly indicate that BCAA inhibits obesity-related liver carcinogenesis by targeting insulin resistance and subsequently by reducing chronic inflammation and adipokine imbalance (Fig. 2). In addition to the liver, supplementation with BCAA suppressed obesity- and diabetes-related carcinogenesis in the colorectum, and this was also associated with the improvement of insulin resistance and inhibition of the activation of

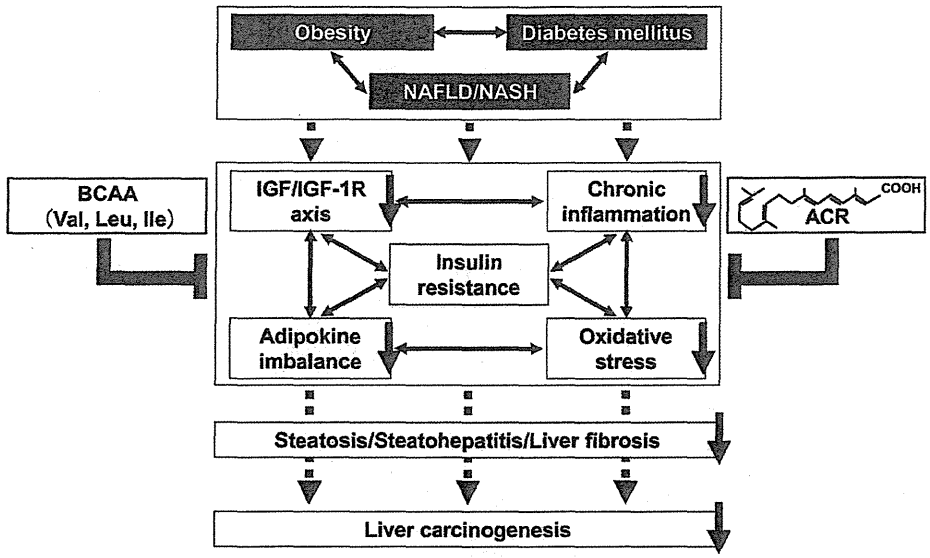

Figure 2. The mechanisms of action of ACR and BCAA in the inhibition of obesity-related liver carcinogenesis. Obesity and diabetes mellitus significantly increase the risk of HCC. Nonalcoholic fatty liver disease and nonalcoholic steatohepatitis, which are usually associated with obesity and diabetes, also play critical roles in the development of HCC. Several pathophysiological mechanisms link obesity and liver carcinogenesis, including the emergence of insulin resistance, activation of the IGF/IGF-1R axis, a state of chronic inflammation, induction of oxidative stress, and occurrence of adipokine imbalance. Among them, in particular, insulin resistance plays a key role in obesity-related liver carcinogenesis. Oral supplementation with BCAA significantly reduces the risk of HCC development in obese cirrhotic patients, and this might be associated with decreased insulin resistance and hepatic steatosis, inhibition of the activation of the IGF/IGF-1R axis, and attenuation of oxidative stress and hyperleptinemia. ACR administration also prevents obesity- and diabetes-related liver tumorigenesis in mice by improving hepatic steatosis and insulin resistance, while attenuating chronic inflammation.

the IGF/IGF-IR axis [123]. BCAA, therefore, may be a useful chemoprevention modality for HCC and probably colorectal cancer in obese people.

13 Conclusion

Throughout this review, we have indicated that both ACR and BCAA are promising agents for the prevention of liver carcinogenesis. Therefore, we considered that a combination therapy involving both ACR and BCAA may better inhibit HCC cell growth. Interestingly, a combined ACR and BCAA treatment significantly inhibited the growth of human HCC xenografts in nude mice by inhibiting the phosphorylation of the RXRα, ERK, Akt, and IGF-1R proteins in the xenografts [124]. These results indicated that this combination might be effective for the treatment and probably chemoprevention of HCC. The beneficial effects of the combination approach to chemoprevention, using ACR as a key agent for the prevention and treatment of HCC, have been previously reported [58, 59, 125–127]. A clinical trial also demonstrated that the combination of BCAA and perindopril, an antihypertensive drug, inhibited the cumulative recurrence of HCC after curative therapy and this was associated with improved insulin resistance [128]. Therefore, a combination therapy using ACR and/or BCAA may represent a potential new strategy for chemoprevention of HCC development.

In summary, the poor prognosis of patients with HCC is because of its high incidence and recurrence in cirrhotic livers. Therefore, more effective strategies for the chemoprevention of HCC should be developed to directly improve prognoses for these patients. The results from both experimental and clinical studies strongly suggest that pharmaceutical and nutraceutical approaches, in particular using ACR and BCAA, play a central role in this strategy. These agents may also play a critical role in the prevention of obesity-related liver carcinogenesis, which is a new, serious problem in modern society.

The authors have declared no conflict of interest.

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