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Synergistic growth inhibition by acyclic retinoid and phosphatidylinositol 3-kinase inhibitor in human hepatoma cells

Atsushi Baba, Masahito Shimizu*, Tomohiko Ohno, Yohei Shirakami, Masaya Kubota, Takahiro Kochi, Daishi Terakura, Hisashi Tsurumi and Hisataka Moriwaki

Abstract

Background: A malfunction of RXRα due to phosphorylation is associated with liver carcinogenesis, and acyclic retinoid (ACR), which targets RXRα, can prevent the development of hepatocellular carcinoma (HCC). Activation of PI3K/Akt signaling plays a critical role in the proliferation and survival of HCC cells. The present study examined the possible combined effects of ACR and LY294002, a PI3K inhibitor, on the growth of human HCC cells.

Methods: This study examined the effects of the combination of ACR plus LY294002 on the growth of HLF human HCC cells.

Results: ACR and LY294002 preferentially inhibited the growth of HLF cells in comparison with Hc normal hepatocytes. The combination of 1 μM ACR and 5 μM LY294002, in which the concentrations used are less than the IC_{50} values of these agents, synergistically inhibited the growth of HLF, Hep3B, and Huh7 human HCC cells. These agents when administered in combination acted cooperatively to induce apoptosis in HLF cells. The phosphorylation of RXRα, Akt, and ERK proteins in HLF cells were markedly inhibited by treatment with ACR plus LY294002. Moreover, this combination also increased RXRE promoter activity and the cellular levels of RARβ and $p21^{CIP1}$, while decreasing the levels of cyclin D1.

Conclusion: ACR and LY294002 cooperatively increase the expression of RARβ, while inhibiting the phosphorylation of RXRα, and that these effects are associated with the induction of apoptosis and the inhibition of cell growth in human HCC cells. This combination might therefore be effective for the chemoprevention and chemotherapy of HCC.

Keywords: Acyclic retinoid, LY294002, Hepatocellular carcinoma, RXRα, Synergism

Background

Retinoids, vitamin A metabolites and analogs, are ligands of the nuclear receptor superfamily that exert fundamental effects on cellular activities, including growth, differentiation, and death (regulation of apoptosis). Retinoids exert their biological functions primarily by regulating gene expression through 2 distinct nuclear receptors, the retinoic acid receptors (RARs) and retinoid X receptors (RXRs), which are ligand-dependent transcription factors [1,2]. Among retinoid receptors, RXRs are regarded as master regulators of the nuclear receptor superfamily because they play an essential role in controlling normal cell proliferation and metabolism by acting as common heterodimerization partners for various types of nuclear receptors [1,2]. Therefore, altered expression and function of RXRs are strongly associated with the development of various disorders, including cancer, whereas targeting RXRs by retinoids might be an effective strategy for the prevention and treatment of human malignancies [3].

Hepatocellular carcinoma (HCC) is one of the most frequently occurring cancers worldwide. Recent studies have revealed that a malfunction of RXRα, one of the subtypes of RXR, due to aberrant phosphorylation by the Ras/mitogen-activated protein kinase (MAPK) signaling pathway is profoundly associated with liver carcinogenesis [4-9]. On the other hand, a prospective randomized study showed that administration of acyclic retinoid (ACR), a

* Correspondence: shimim-gif@umin.ac.jp
Department of Gastroenterology, Gifu University Graduate School of Medicine, Graduate School of Medicine, 1-1 Yanagido, Gifu 501-1194, Japan

synthetic retinoid which targets RXRα, inhibited the development of a second primary HCC, and thus improved patient survival from this malignancy [10,11]. ACR inhibits the growth of HCC-derived cells via the induction of apoptosis by working as a ligand for retinoid receptors [12,13]. ACR also suppresses HCC cell growth and inhibits the development of liver tumors by inhibiting the activation and expression of several types of growth factors and their corresponding receptor tyrosine kinases (RTKs), which lead to the inhibition of the Ras/MAPK activation and RXRα phosphorylation [8,9,14-17]. These reports strongly suggest that ACR might be a promising agent for the prevention and treatment of HCC.

Phosphatidylinositol 3-kinase (PI3K) is activated by growth factor stimulation through RTKs and Ras activation, and plays a critical role in cell survival and proliferation in collaboration with its major downstream effector Akt, a serine-threonine kinase [18-20]. Increasing evidence has shown that aberrant activation of the PI3K/Akt pathway is implicated in the initiation and progression of several types of human malignancies, including HCC, indicating that targeting PI3K/Akt signaling might be an effective strategy for the treatment of cancers [18-22]. Several clinical trials have been conducted to investigate the safety and anti-cancer effects of therapeutic agents that inhibit the PI3K/Akt signaling cascade [18-20]. Combined treatment with a PI3K/Akt inhibitor and other agents, including MAPK inhibitors, might also be a promising regimen that exerts potent anti-cancer properties [23,24].

Combination therapy and prevention using ACR as a key drug is promising for HCC treatment because ACR can act synergistically with other agents in suppressing growth and inducing apoptosis in human HCC-derived cells [17,25-30]. The aim of the present study is to investigate whether the combination of ACR plus LY294002, a PI3K inhibitor, exerts synergistic growth inhibitory effects on human HCC cells, and to examine possible mechanisms for such synergy, predominantly focusing on the inhibitory effects on RXRα phosphorylation by a combination of these agents.

Methods

Materials

ACR (NIK-333) was supplied by Kowa Pharmaceutical (Tokyo, Japan). LY294002 was purchased from Wako (Osaka, Japan). Another PI3K inhibitor NVP-BKM120 (BKM120) was from Selleck Chemicals (Houston, TX, USA).

Cell lines and cell culture conditions

HLF, Huh7, Hep3B, and HepG2 human HCC cell lines were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan) and were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FCS and 1% penicillin/streptomycin. The Hc human normal hepatocyte cell line was purchased from Cell Systems (Kirkland, WA, USA) and maintained in CS-S complete medium (Cell Systems). These cells were cultured in an incubator with humidified air containing 5% CO_2 at 37°C.

Cell proliferation assays

Three thousand HCC (HLF, Huh7, Hep3B, and HepG2) or Hc cells were seeded on 96-well plates in serum-free medium. Twenty-four hours later, the cells were treated with the indicated concentrations of ACR or LY294002 for 48 hours in DMEM supplemented with 1% FCS. Cell proliferation assays were performed using a MTS assay (Promega, Madison, WI, USA) according to the manufacturer's instructions. The combination index (CI)-isobologram was used to determine whether the combined effects of ACR plus LY294002 were synergistic [25,27,30,31]. HLF cells were also treated with a combination of the indicated concentrations of ACR and BKM120 for 48 hours to examine whether this combination synergistically inhibited the growth of these cells.

Apoptosis assays

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) and caspase-3 activity assays were conducted to evaluate apoptosis. For the TUNEL assay, HLF cells (1×10^6), which were treated with 1 μM ACR alone, 5 μM LY294002 alone, or a combination of these agents for 48 hours, were stained with TUNEL methods using an In Situ Cell Death Detection Kit, Fluorescein (Roche Diagnostics, Mannheim, Germany) [25]. The caspase-3 activity assay was performed using HLF cells that were treated with the same concentrations of the test drugs for 72 hours. The cell lysates were prepared and the caspase-3 activity assay was performed using an Apoalert Caspase Fluorescent Assay Kit (Clontech Laboratories, Mountain View, CA, USA) [30].

Protein extraction and western blot analysis

Protein extracts were prepared from HLF cells treated with 1 μM ACR alone, 5 μM LY294002 alone, or a combination of these agents for 12 hours because this treatment time was appropriate for evaluating the expression levels of phosphorylated extracellular signal-regulated kinase (p-ERK), phosphorylated Akt (p-Akt), and phosphorylated RXRα (p-RXRα) proteins [25,29,30]. Equivalent amounts of extracted protein were examined by western blot analysis using specific antibodies [25]. The anti-RXRα and anti-RARβ antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The primary antibodies for ERK, p-ERK, Akt, p-Akt, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were from Cell Signaling Technology (Beverly, MA, USA). The antibody for p-RXRα was kindly provided by Drs. S. Kojima

and H. Tatsukawa (RIKEN Advanced Science Institute, Saitama, Japan).

RNA extraction and quantitative RT-PCR analysis

Total RNA was isolated from HLF cells using an RNAqueous-4PCR kit (Ambion Applied Biosystems, Austin, TX, USA) and cDNA was amplified from 0.2 μg of total RNA using the SuperScript III Synthesis system (Invitrogen, Carlsbad, CA, USA) [32]. Quantitative real-time reverse transcription PCR (RT-PCR) analysis was performed using specific primers that amplify the RARβ, $p21^{CIP1}$, cyclin D1, and β-actin genes. The specific primer sets used have been described elsewhere [25,30].

RXRE reporter assays

HLF cells were transfected with RXR-response element (RXRE) reporter plasmids (100 ng/well in 96-well dish), which were kindly provided by the late Dr. K. Umesono (Kyoto University, Kyoto, Japan), along with p*RL*-CMV (*Renilla* luciferase, 10 ng/well in 96-well dish; Promega) as an internal standard to normalize transfection efficiency. Transfections were carried out using Lipofectamine LTX Reagent (Invitrogen). After exposure of cells to the transfection mixture for 24 hours, the cells were treated with 1 μM ACR alone, 5 μM LY294002 alone, or a combination of these agents for 24 hours. The cell lysates were then prepared, and the luciferase activity of each cell lysate was determined using a dual-luciferase reporter assay system (Promega) [25].

Statistical analysis

The data are expressed in terms of means ± SD. The statistical significance of the differences in the mean values was assessed using one-way ANOVA, followed by Tukey-Kramer multiple comparison tests. Values of <0.05 were considered significant.

Results

ACR and LY294002 cause preferential inhibition of growth in HLF human HCC cells in comparison with Hc normal hepatocytes

In the initial study, the growth inhibitory effect of ACR and LY294002 on HLF human HCC cells and on Hc hepatocytes was examined. ACR (Figure 1A) and LY294002 (Figure 1B) inhibited the growth of HLF cells with IC_{50} values of approximately 6.8 μM and 15 μM, respectively. On the other hand, Hc cells were resistant to these agents because the IC_{50} values of ACR and LY294002 for the growth inhibition of Hc cells were each greater than 50 μM (Figure 1). These results suggest that ACR and LY294002 preferentially inhibit the growth of HCC cells compared with that of normal hepatocytes.

ACR along with LY294002 causes synergistic inhibition of growth in HCC cells

Next, the effects of the combined treatment of ACR plus LY294002 on the growth of HCC-derived cells and Hc hepatocytes were examined. When HLF human HCC cells were treated with a range of concentrations of these agents, the CI indices for less than 1 μM ACR (0.5 or 1 μM) plus less than 10 μM LY294002 (5 or 10 μM) were 1+ (slight synergism), 2+ (moderate synergism), or 3+ (synergism). In particular, the combination of as little as 1 μM ACR (approx. IC_{15} value) and 5 μM LY294002 (approx. IC_{25} value) exerted synergistic growth inhibition because the CI-isobologram analysis yielded a CI index of 0.54 (3+), which indicates synergism [25,27,30,31], with this combination (Figure 2A,B, and Table 1). In other HCC cell lines, including Huh7, Hep3B, and HepG2 cell lines, similar findings were also obtained using Huh7 and Hep3B cells; the combination of 1 μM ACR plus 5 μM LY294002 significantly suppressed the growth of these cells (Figure 2C). In contrast, the growth of Hc normal hepatocytes was not affected by the combination of these agents; even a

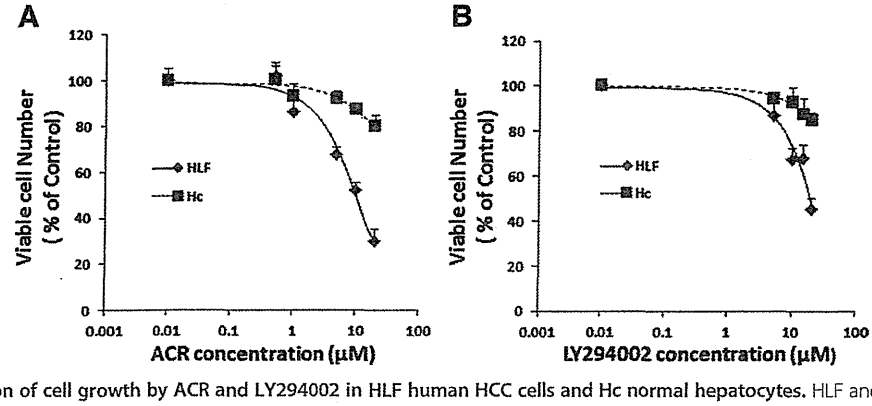

Figure 1 Inhibition of cell growth by ACR and LY294002 in HLF human HCC cells and Hc normal hepatocytes. HLF and Hc cells were treated with the indicated concentrations of ACR **(A)** or LY294002 **(B)** for 48 hours. Cell viability was determined by the MTS assay and expressed as a percentage of the control value. Error bars present the SD of triplicate assays.

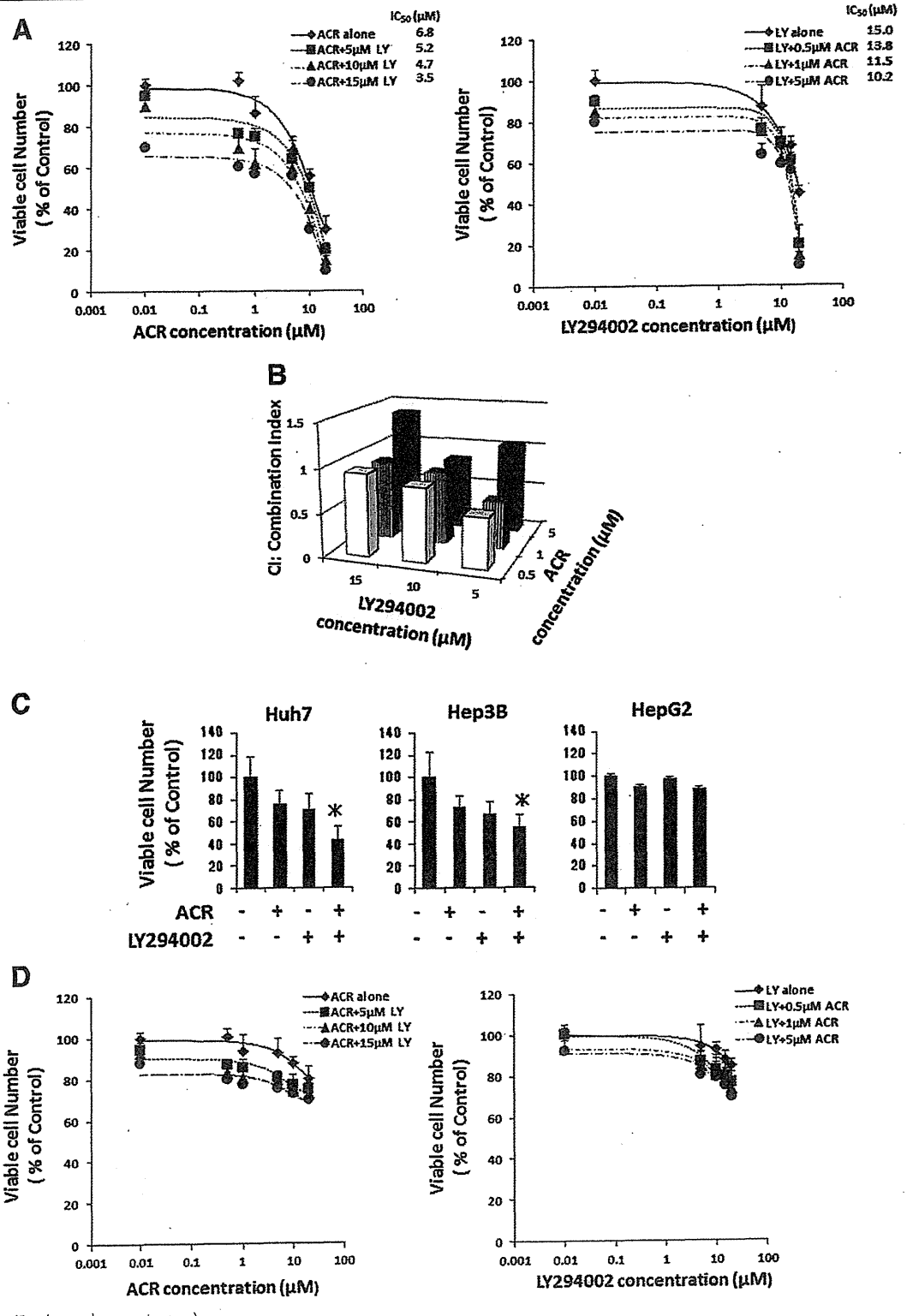

Figure 2 (See legend on next page.)

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Figure 2 Inhibition of cell growth by ACR alone, LY294002 alone, or various combinations of these agents in human HCC-derived cells and Hc normal hepatocytes. **(A)** HLF human HCC cells were treated with the indicated concentrations of ACR alone, LY294002 alone, and various combinations of these agents for 48 hours. **(B)** The data obtained in **(A)** was used to calculate the combination index. **(C)** Huh7, Hep3B, and HepG2 human HCC cells were treated with vehicle, 1 μM ACR alone, 5 μM LY294002 alone, or a combination of 1 μM ACR and 5 μM LY294002 for 48 hours. **(D)** Hc human hepatocytes were treated with the indicated concentrations of ACR alone, LY294002 alone, and various combinations of these agents for 48 hours. **(A)**, **(C)**, and **(D)** Cell viability was determined by the MTS assay and expressed as a percentage of the control value. Error bars present the SD of triplicate assays. * $P < 0.05$.

combination of high concentrations of ACR (5 μM) plus LY294002 (15 μM) did not inhibit the growth of Hc cells in the present study (Figure 2D).

ACR plus BKM120 cause synergistic inhibition of growth in HLF cells

In order to examine whether PI3K inhibitors are promising agents to potently suppress the growth of HCC cells in conjunction with ACR, the combined effects of ACR plus BKM120, another selective PI3K inhibitor [33], on the growth of HLF cells were next investigated. The combination of ACR plus BKM120 significantly inhibited the growth of HLF cells. In particular, when the cells were treated with 1 μM ACR plus 5 μM BKM120, the CI-isobologram analysis yielded a CI-index of 3+ (synergism) (Figure 3A,B, and Table 1). These findings suggest that combination therapy using ACR plus PI3K inhibitors might be an effective regimen for inhibiting the growth of HCC cells.

ACR plus LY294002 cooperatively induce apoptosis in HLF cells

The next study examined whether the synergistic growth inhibition in HLF cells induced by treatment with ACR plus LY294002 is associated with the induction of apoptosis. The ratio of TUNEL-positive cells was not significantly increased by treatment with 1 μM ACR alone (26.9%) or 5 μM LY294002 alone (27.6%) in comparison to that of control untreated cells (15.2%). However, when the cells were treated with the combination of these agents, TUNEL-positive cells significantly increased to 54.4% of the total remaining cells (Figure 4A). Similar results were also observed in the caspase-3 activity assay; the combined treatment with ACR plus LY294002 significantly increased the levels of caspase-3 activity in HLF cells, whereas treatment with ACR alone or LY294002 alone did not exert such an effect (Figure 4B).

ACR plus LY294002 cooperatively suppress the phosphorylation of RXRα, ERK, and Akt and increase the RXRE promoter activity in HLF cells

RXRα phosphorylation is involved in the development of HCC, and thus might be a promising target for HCC chemoprevention [4-9]. Therefore, the effects of the combination of ACR and LY294002 on the phosphorylation of RXRα and related signaling molecules were next investigated in HLF cells. As shown in Figure 5A, there was a significant decrease in the expression levels of p-RXRα, p-ERK, and p-Akt proteins when the cells were treated with 1 μM ACR. Treatment with 5 μM LY294002 also caused a marked decrease in the expression levels of p-RXRα and p-Akt proteins in these cells. Moreover, the decrease in the expression levels of p-RXRα, p-ERK, and p-Akt proteins was greater when the cells were treated with a combination of these agents.

Table 1 Combined effects of ACR and PI3K inhibitors on HLF cells

ACR concentration (μM)	LY294002 concentration (μM)			BKM120 concentration (μM)		
	5	10	15	5	10	15
0.5	+++	+	±	±	++	++
1	+++	++	±	+++	++	+
5	-	++	-	-	-	-

Note:
"-", CI1.1-1.3 moderate antagonism;
"±", CI0.9-1.1 additive effect;
"+", CI0.8-0.9 slight synergism;
"++", CI0.6-0.8 moderate synergism;
"+++", CI0.4-0.6 synergism;
Abbreviations: CI Combination index, ACR Acyclic retinoid.

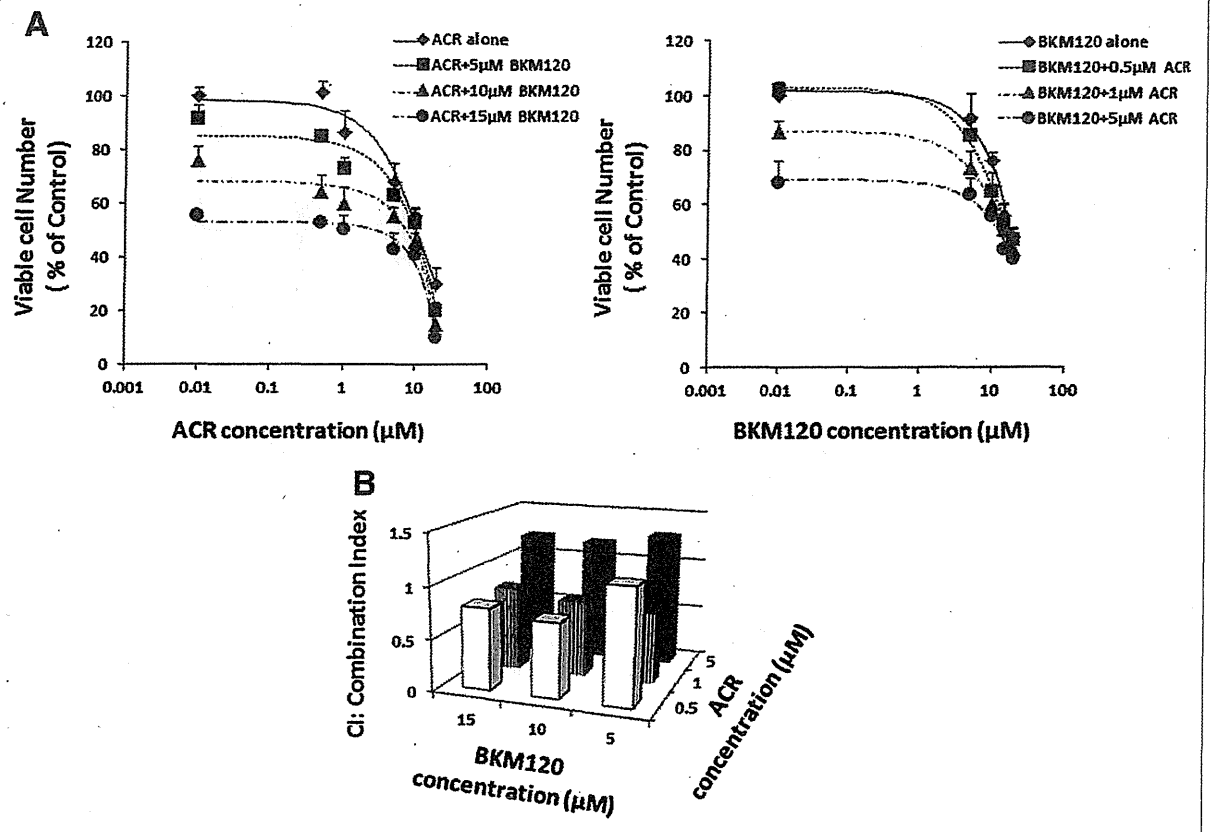

Figure 3 Inhibition of cell growth by ACR alone, BKM120 alone, or various combinations of these agents in HCC cells. (A) HLF human HCC cells were treated with the indicated concentrations of ACR alone, BKM120 alone, or various combinations of these agents for 48 hours. Cell viability was determined by the MTS assay and expressed as a percentage of the control value. **(B)** The data obtained in **(A)** was used to calculate the combination index. Error bars present the SD of triplicate assays.

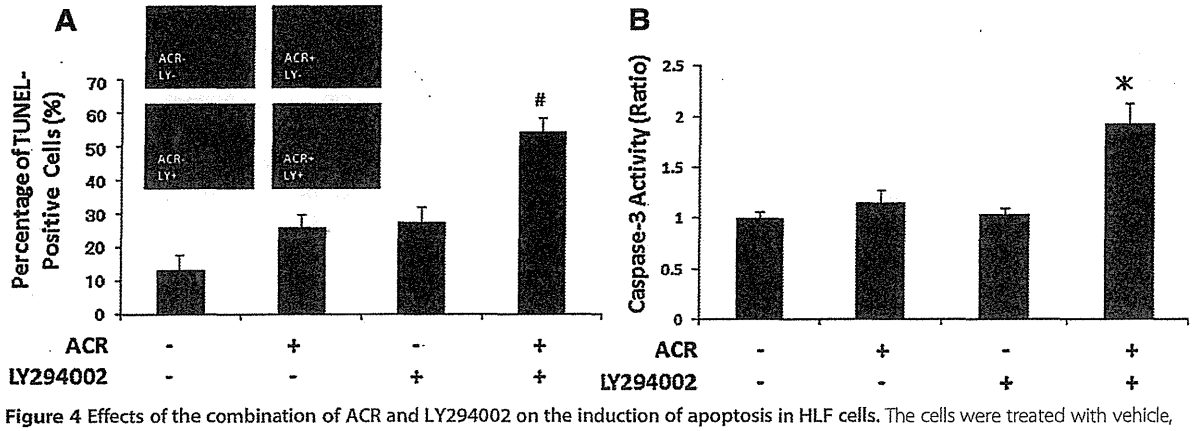

Figure 4 Effects of the combination of ACR and LY294002 on the induction of apoptosis in HLF cells. The cells were treated with vehicle, 1 μM ACR alone, 5 μM LY294002 alone, or a combination of 1 μM ACR and 5 μM LY294002 for 48 or 72 hours. **(A)** TUNEL assays were performed using cells treated with test drugs for 48 hours. TUNEL-positive cells were counted and examined as the percentage of the DAPI-positive cell number (500 cells were counted in each flask). **(B)** Caspase-3 activity assays were performed with a fluorometric system using samples treated for 72 hours. # $P < 0.01$. * $P < 0.05$.

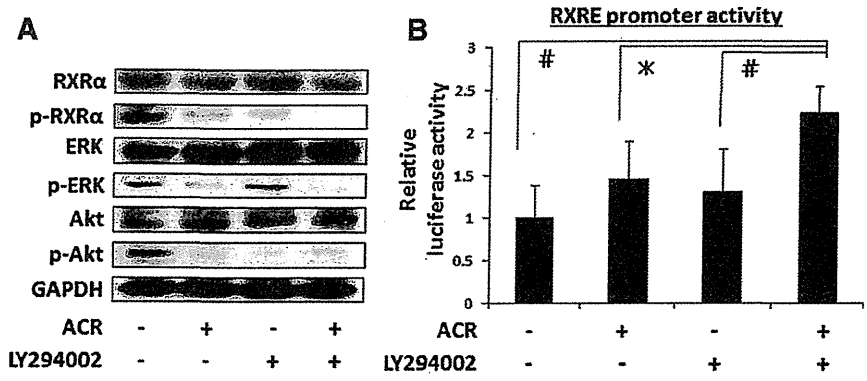

Figure 5 Effects of the combination of ACR and LY294002 on the phosphorylation of RXRα, ERK, and Akt proteins and the transcriptional activity of the RXRE promoter in HLF cells. (A) The cells were treated with vehicle, 1 μM ACR alone, 5 μM LY294002 alone, or a combination of 1 μM ACR and 5 μM LY294002 for 12 hours. The extracted proteins were examined by western blot analysis using the respective antibodies. Repeat western blots gave similar results. **(B)** A transient transfection reporter assay was performed with the RXRE luciferase reporter in the presence of vehicle, 1 μM ACR alone, 5 μM LY294002 alone, or a combination of 1 μM ACR and 5 μM LY294002. Relative luciferase activity was determined after 24 hours. Columns and lines indicate the means and SD of triplicate assays. # $P < 0.01$. * $P < 0.05$.

In addition, there was a significant increase in the transcriptional activity of the RXRE reporter when HLF cells were treated with a combination of ACR and LY294002, whereas treatment with the same concentrations of ACR alone or LY294002 alone did not upregulate the activity of this promoter (Figure 5B). Because RXRs modulate the expression of target genes by interacting with the RXRE element located in the promoter regions of these genes [1,2], this finding may indicate that LY294002 enhances the transcriptional activity of the RXRE promoter induced by ACR, at least in part by inhibiting the phosphorylation of RXRα.

ACR and LY294002 cooperatively increase the cellular levels of RARβ and $p21^{CIP1}$, but decrease the levels of cyclin D1, in HLF cells

Because the transcriptional activity of the RXRE promoter was significantly increased by treatment with ACR plus LY294002 (Figure 5B), the next study examined whether this combination cooperatively altered the expression of target molecules of ACR, including RARβ, $p21^{CIP1}$, and cyclin D1 [13,25,27,34], in HLF cells. As shown in Figure 6A, the mRNA and protein expression levels of RARβ were significantly increased on combined treatment with ACR and LY294002. Quantitative RT-PCR analyses also revealed that there was a significant increase in the levels of $p21^{CIP1}$ mRNA, but a decrease in the levels of cyclin D1 mRNA, in HLF cells, upon treatment with this combination (Figure 6B).

Discussion and conclusions

In order to improve the clinical outcome for patients with HCC, development of effective strategies for the chemoprevention and chemotherapy of this malignancy is urgently required. We believe that combination chemoprevention using ACR as a key agent is a promising method for attaining this objective, because it provides an opportunity to take advantage of the synergistic effects of ACR on growth inhibition in HCC cells [17,25-30]. The present study provides the first evidence that the combination of ACR with LY294002, a PI3K inhibitor, synergistically inhibited the growth of human HCC cells through the induction of apoptosis. Activation of the PI3K/Akt pathway, which is common in many cancers such as HCC [21,22], contributes to the inhibition of apoptosis and induction of therapeutic resistance in cancer cells, indicating that targeting this pathway can inhibit the survival and growth of cancer cells through various mechanisms such as potentiation of the effects of chemotherapeutic drugs [18-20,23,24]. For instance, the combination of all-*trans* retinoic acid with LY294002 enhanced growth suppressive effects in leukemic cells by inducing apoptosis [35].

The hypotheses that explain the synergism generated by the combination of ACR and LY294002 are summarized in Figure 7. First, it should be noted that phosphorylation of RXRα was markedly inhibited by the combination of ACR and LY294002 in the present study. This finding seems to be significant. because RXRα phosphorylation plays a role in the development of HCC and, therefore, might be a critical target for the implementation of HCC chemoprevention [5,7-9]. Accumulation of phosphorylated RXRα induced by the Ras/MAPK activation interferes with the function of normal (unphosphorylated) RXRα in a dominant negative manner [8,9]. This and prior studies [4,17,25,28] show that ACR alone inhibits the phosphorylation of RXRα and ERK proteins in HCC cells. Moreover, in the present study, ACR alone also dephosphorylated the Akt protein in HLF cells. These

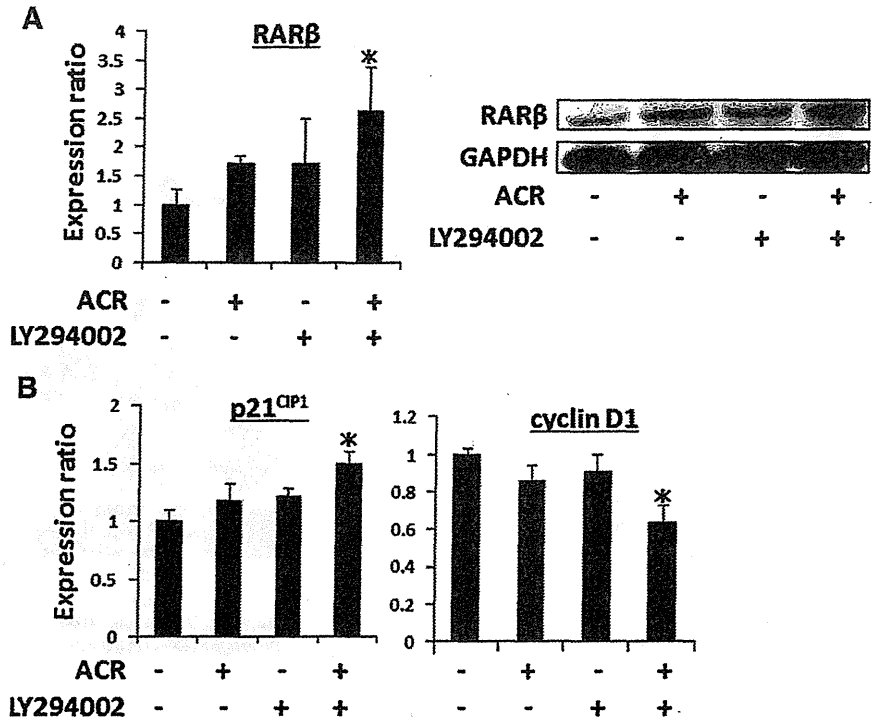

Figure 6 Effects of the combination of ACR and LY294002 on the cellular expression levels of RARβ, $p21^{CIP1}$, and cyclin D1 in HLF cells. **(A)** The expression levels of RARβ mRNA (left panel) and protein (right panel) were examined by quantitative real-time RT-PCR analysis and western blot analysis, respectively, using cells treated with the test drugs for 24 hours. **(B)** Quantitative real-time RT-PCR analysis to examine the expression levels of $p21^{CIP1}$ and cyclin D1 mRNAs were performed using cells treated with the test drugs for 24 hours. The expression level of each mRNA was normalized to the level of β-actin mRNA. Values represent the means ± SD of triplicate analyses. * $P < 0.05$.

findings suggest that the combination of ACR and LY294002 cooperatively inhibit the phosphorylation of RXRα through dephosphorylation of ERK and Akt, which leads to the synergistic inhibition of growth and the induction of apoptosis in HCC cells. The results of the present research, together with those of previous studies [17,25,28-30], suggest that dephosphorylation of RXRα might be a key mechanism for ACR-based combination chemoprevention in HCC cells.

Phosphorylated RXRα loses its ability to form heterodimers with RARβ and this is associated with resistance to retinoids [7]. Therefore, restoration of the function of RXRα by inhibiting its phosphorylation is critical to regulate the expression of retinoid target genes [4-9]. In comparison to treatment with ACR alone or LY294002 alone, combined treatment with these agents significantly increased the transcriptional activity of the RXRE reporter in the present study. This combination also significantly altered the expression levels of ACR target genes, such as RARβ, $p21^{CIP1}$, and cyclin D1 mRNA [13,25,27,34]. Particularly, the induction of RARβ by the combination of ACR and LY294002 might play a crucial role in inhibiting the growth of HCC cells because RARβ, which is a receptor for ACR [36], can exert tumor-suppressive effects in cancer cells and thus be considered as a tumor suppressor gene [37].

In this study, the phosphorylation of Akt is inhibited by ACR alone in HLF cells. This finding seems to be of interest because Akt phosphorylation plays a critical role in cell survival, prevention of apoptosis, and progression of cell cycle in various types of tumors, including HCC [21,22]. The precise mechanism by which ACR inhibits the phosphorylation of Akt protein has not been determined. However, we assume that the dephosphorylation of this protein by ACR might be explained by, at least in part, its ability to inhibit growth factor-dependent RTK activity, because Akt is potently phosphorylated by the activation of RTKs [8,9,14,15,18-20]. For instance, ACR inhibits the growth of HCC cells and prevents chemically induced liver tumorigenesis by targeting the transforming growth factor-α/epidermal growth factor receptor (EGFR) axis, which belongs to RTKs [14,15]. Moreover, a recent study showed that retinol inhibited PI3K activity by decreasing the interaction between PI3K and phosphatidylinositol and this was associated with suppression of cell growth in colon cancer cells [38]. These studies suggest that the PI3K/Akt signaling pathway might be a critical target for retinoids to exert their anti-cancer and chemopreventive properties.

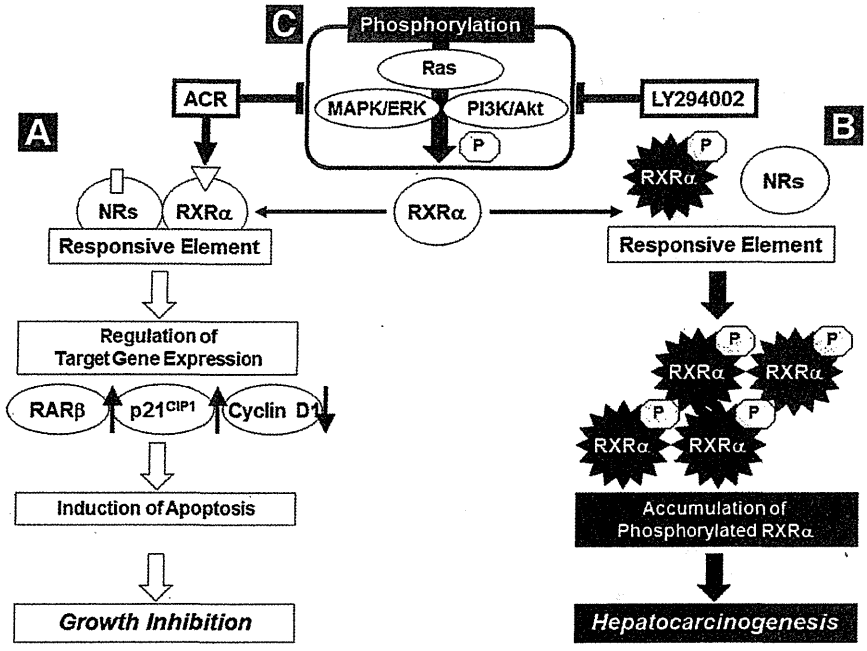

Figure 7 A hypothetical schematic representation of the effects of the combination of ACR and LY294002 on growth inhibition in HCC cells. When ACR binds to and activates RXRα, it forms homo- and/or heterodimers with other nuclear receptors (NRs), including RARs. This results in the activation of the transcriptional activity of the responsive element, thus controlling the expression of the target genes, such as RARβ, $p21^{CIP1}$, and cyclin D1, which induce apoptosis and inhibit the growth of HCC cells **(A)**. In HCC cells, the MAPK/ERK and PI3K/Akt pathways, both of which are located downstream of Ras, are highly activated and phosphorylate the RXRα protein. The accumulation of phosphorylated RXRα protein, which impairs dimer formation and the subsequent transactivation functions of this receptor, cause a deviation from normal cell proliferation and differentiation, thereby playing a critical role in liver carcinogenesis **(B)**. ACR and LY294002 inhibit RXRα phosphorylation by inhibiting ERK and Akt phosphorylation, resulting in restoration of receptor function and activation of the transcriptional activity of the responsive element **(C)**. For additional details, see the Discussion section.

In the current study, the combination of ACR and LY294002 significantly inhibited the growth of HLF, Huh7, and Hep3B HCC cells, whereas the growth of HepG2 cells, the other HCC cell line, was not suppressed by this combination. This might be associated with the phosphorylation status of ERK and Akt proteins because the expression levels of p-ERK and p-Akt proteins were increased in HLF, Huh7, and Hep3B cells compared with HepG2 cells [29]. These results, on the other hand, suggest that HCC cells that overexpress p-ERK and p-Akt proteins might be more sensitive targets for combination therapy using ACR and PI3K inhibitors.

Finally, it should be emphasized that combination therapy and prevention are advantageous because, in addition to providing the potential for synergistic effects, they may reduce the opportunity for the development of drug resistance by cancer cells. Several preclinical studies have shown that cancer cells harboring activated Ras mutations appear to be resistant to treatment with PI3K inhibitor alone [23,39]. However, the use of a combination of the PI3K/Akt inhibitor and a MAPK inhibitor significantly exerted anti-cancer effects in *Kars* G12D-driven or EGFR-mutant lung tumors [23,24]. These studies suggest that effective treatment with PI3K inhibitors require concomitant therapies that target RTK/Ras/MAPK signaling and, therefore, ACR, which can inhibit this signaling pathway [8,9,14,15,40], might be a preferable partner for PI3K inhibitors.

In conclusion, the present study indicates that the combination of ACR and LY294002, which can inhibit the phosphorylation of RXRα, causes a synergistic induction of apoptosis and inhibition of cell growth in human HCC cells. The results of our study suggest that this combination might hold promise as a clinical modality for the prevention and treatment of HCC, due to their synergistic effects. In particular, our finding that the combination regimen using 1 μM ACR plus 5 μM LY294002 synergistically inhibits the growth of HCC cells seems to be clinically relevant because this concentration (1 μM) is approximately the same as the plasma concentration of ACR (which ranged from 1 to 5 μM) in a clinical trial that demonstrated the chemopreventive effects of this agent in the recurrence of secondary HCC [10,11].

Abbreviations

ACR: Acyclic retinoid; CI: Combination index; DMEM: Dulbecco's modified eagle medium; EGFR: Epidermal growth factor receptor; ERK: Extracellular signal-regulated kinase; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; HCC: Hepatocellular carcinoma; IFN: Interferon; MAPK: Mitogen-activated protein kinase; PI3K: Phosphatidylinositol 3-kinase; RAR: Retinoic acid receptor; RTK: Receptor tyrosine kinase; RT-PCR: Reverse transcription PCR; RXR: Retinoid X receptor; RXRE: Retinoid X receptor response element; TUNEL: Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

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

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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

Takahiro Kochi [a], Masahito Shimizu [a,*], Daishi Terakura [a], Atsushi Baba [a], Tomohiko Ohno [a], Masaya Kubota [a], Yohei Shirakami [a], Hisashi Tsurumi [a], Takuji Tanaka [b], Hisataka Moriwaki [a]

[a] Department of Medicine, Gifu University Graduate School of Medicine, Gifu, Japan
[b] Department of Tumor Pathology, Gifu University Graduate School of Medicine, Gifu, Japan

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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

* Corresponding author. Address: Department of Gastroenterology, Gifu University Graduate School of Medicine, 1-1 Yanagido, Gifu 501-1194, Japan. Tel.: +81 58 230 6313; fax: +81 58 230 6310.
E-mail address: shimim-gif@umin.ac.jp (M. Shimizu).

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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

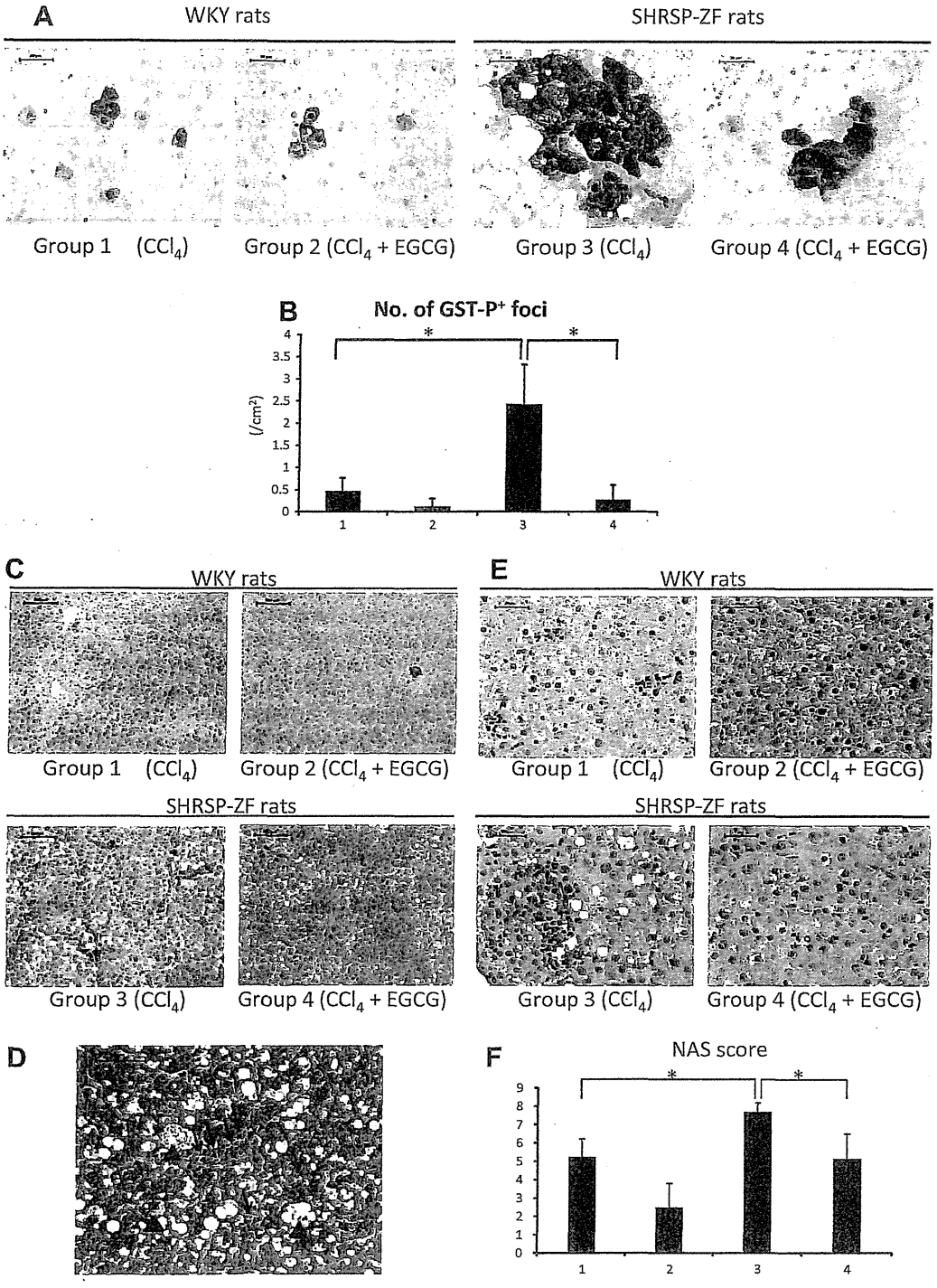

Fig. 1. Effects of EGCG on the development of GST-P$^+$ foci and histopathology in the livers of the experimental rats. (A) Representative photomicrographs of GST-P$^+$ foci and (B) the average number of GST-P$^+$ foci that developed in the livers of the experimental rats. Group 1: WKY rats treated without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

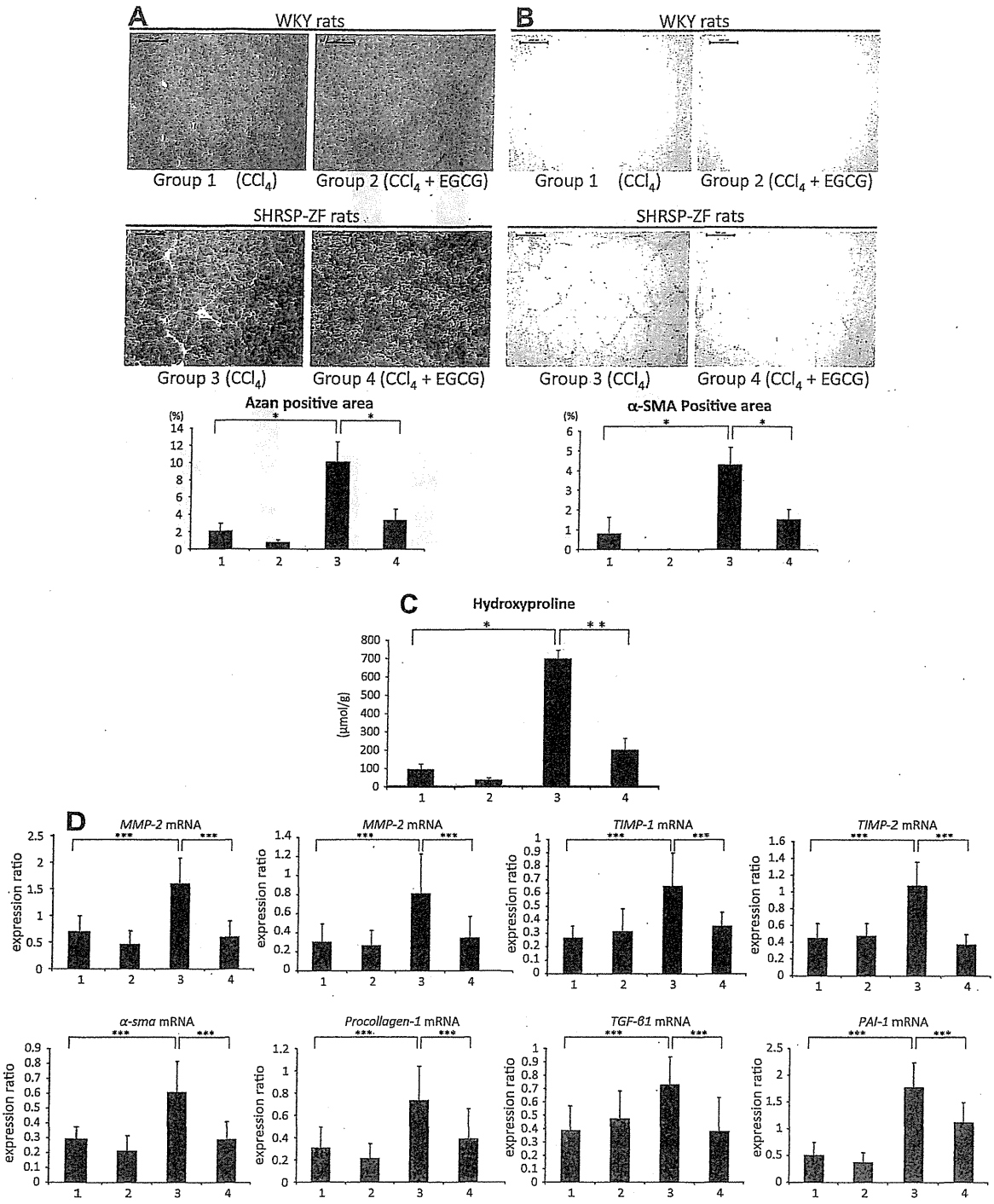

Fig. 2. Effects of EGCG on hepatic fibrosis in the experimental rats. (A) Representative photomicrographs of liver sections stained with Azan stain to show fibrosis (upper panels). The hepatic fibrosis area was evaluated by Azan stain (lower panel). (B) Immunohistochemical detection of α-SMA expression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

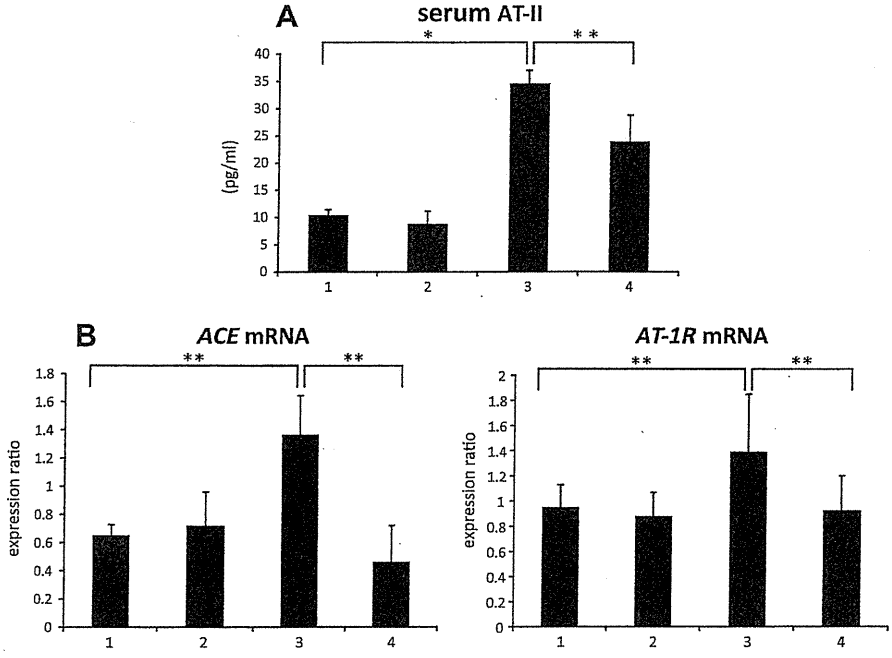

Fig. 3. Effects of EGCG on renin-angiotensin system in the experimental rats. (A) The serum concentrations of AT-II were measured using enzyme immunoassay. (B) The expression levels of ACE and AT-1R mRNA in the livers of the experimental rats were examined by quantitative real-time RT-PCR by using specific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

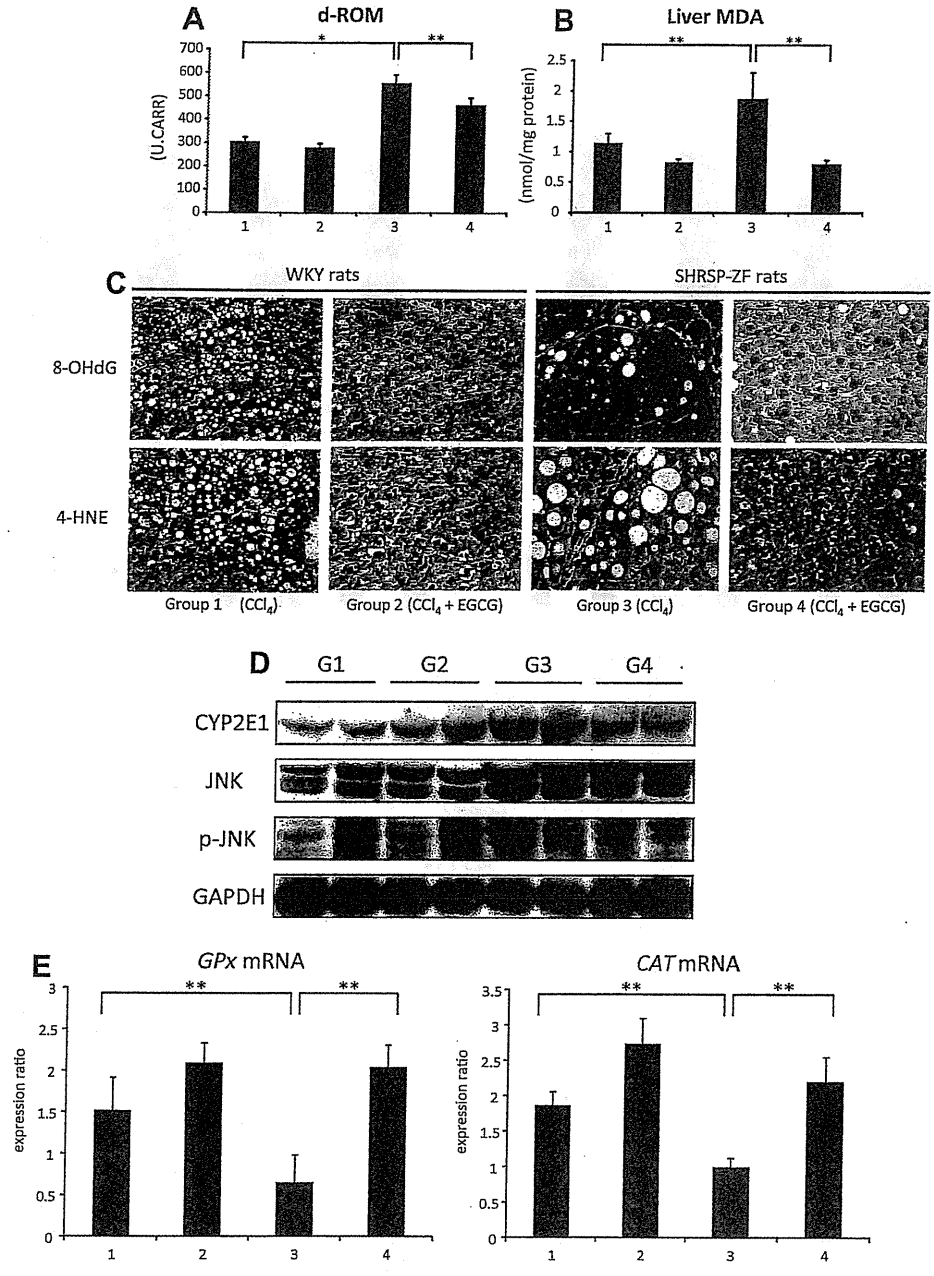

Fig. 4. Effects of EGCG on the serum levels of d-ROM, hepatic concentration of MDA, hepatic expression levels of 8-OHdG, 4-HNE, CYP2E1, JNK, and p-JNK proteins, and hepatic expression levels of GPx and CAT mRNA in the experimental rats. (A) Hydroperoxide levels in the serum were determined by the d-ROM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

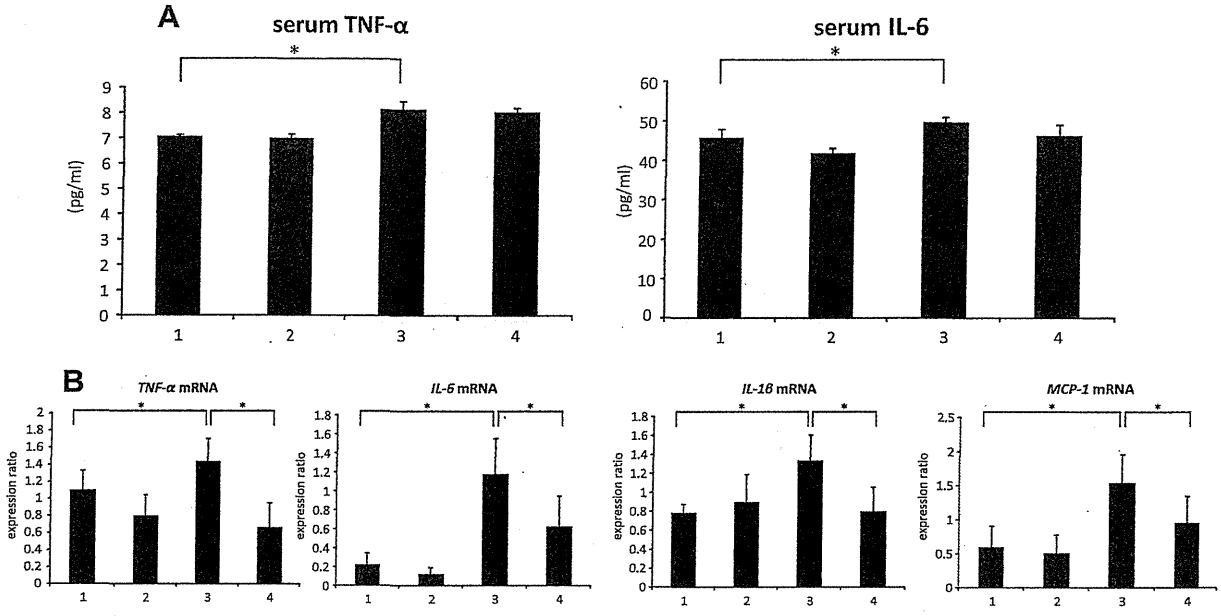

Fig. 5. Effects of EGCG on the serum levels of TNF-α and IL-6 and the expression levels of TNF-α, IL-6, IL-1β, and MCP-1 mRNA in the livers of the experimental rats. (A) The serum concentrations of TNF-α and IL-6 were measured by enzyme immunoassay. (B) Total RNA was isolated from the livers of experimental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