Original Article

Increased levels of serum leptin are a risk factor for the recurrence of stage I/II hepatocellular carcinoma after curative treatment

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Obesity and related adipocytokine disbalance increase the risk of hepatocellular carcinoma. To determine the impact of increased levels of leptin, an obesity-related adipocytokine, on the recurrence of hepatocellular carcinoma, we conducted a prospective case-series analysis. Eighty-five consecutive primary hepatocellular carcinoma patients at our hospital from January 2006 to December 2008 were analyzed. Serum leptin level significantly correlated with Body Mass Index, total body fat, and the amount of subcutaneous fat. They included 33 with stage I/II, who underwent curative treatment. The factors contributing to recurrence of hepatocellular carcinoma, including leptin, were subjected to univariate and multivariate analyses using the Cox proportional hazards model. Body Mass Index ($p = 0.0062$), total body fat ($p = 0.0404$), albumin ($p = 0.0210$), α-fetoprotein ($p = 0.0365$), and leptin ($p = 0.0003$) were significantly associated with the recurrence of hepatocellular carcinoma in univariate analysis. Multivariate analysis suggested that leptin (hazard ratio 1.25, 95% CI 1.07–1.49, $p = 0.0035$) was a sole independent predictor. Kaplan-Meier analysis showed that recurrence-free survival was lower in patients with greater serum leptin concentrations (>5 ng/mL, $p = 0.0221$). These results suggest that the serum leptin level is a useful biomarker for predicting the early recurrence of hepatocellular carcinoma.

Key Words: **hepatocellular carcinoma, carcinogenesis, leptin, obesity, insulin resistance**

Hepatocellular carcinoma (HCC) is the fifth most common malignancy worldwide and is estimated to cause approximately 500,000 deaths annually.[1] HCC frequently develops and in many cases recurs in cirrhotic livers due to persistent hepatitis B virus (HBV) and hepatitis C virus (HCV) infection; this is strongly associated with poor prognosis for this particular malignancy.[2] Therefore, careful surveillance of high-risk groups for HCC is important to improve prognosis. Hence, there is a critical need to identify useful risk factors for the development of HCC. Infection with HBV and HCV, alcohol consumption, aflatoxin exposure, and immune-related hepatitis are accepted as significant risk factors for the development of primary HCC.[3] Male gender, the presence of cirrhosis, high α-fetoprotein (AFP), large tumor foci, multiplicity of tumors, pathologically high-grade atypia of tumor cells, and the presence of portal venous invasion of tumors also raise the risk for HCC recurrence.[4–8]

In addition to these factors, recent studies demonstrate that obesity[9] and related metabolic abnormalities—especially diabetes mellitus (DM) and insulin resistance[10,11]—are important risk factors for the development of HCC. For instance, insulin resistance significantly raises the risk of the recurrence of stage I HCC after curative treatment.[10] Several pathophysiological mechanisms linking obesity and HCC development have been proposed and include the emergence of insulin resistance and a state of chronic inflammation.[12,13] Adipocytokine disbalance might also be involved in obesity-related liver carcinogenesis.[14] Among the adipocytokines, it is well known that the serum levels of leptin, which regulate the homeostasis of glucose and lipid metabolism,[15] are elevated in obese individuals.[16] In addition, both *in vitro* and *in vivo* studies indicate that leptin might play a role in the development of several types of human malignancies, including HCC.[17–21] These findings suggest that the dysregulation of serum leptin levels may be a critical link between obesity and liver carcinogenesis. However, whether leptin is a significant biomarker for predicting the development and/or recurrence of HCC has not been explored.

In this study, we measured the serum leptin concentration in patients with HCC and examined whether it is correlated with obesity and insulin resistance. In addition, we designed a prospective case-series analysis to examine the recurrence-free survival in consecutive patients with stage I/II HCC, who received curative treatment by surgical resection or radiofrequency ablation (RFA), stratified by serum leptin concentrations.

Materials and Methods

Patients. From January 2006 to December 2008, 85 primary HCC patients underwent initial treatment at our hospital. We measured visceral and subcutaneous fat volume using computed tomography (CT) scans at the umbilical level according to a previously reported technique (fatAnalyses and EV Insite R, PSP Corporation, Tokyo, Japan).[22] Tumor stage was defined according to the staging system of the Liver Cancer Study Group of Japan (LCSGJ).[23] HCC nodules were detected by imaging modalities including abdominal ultrasonography (US), dynamic CT, dynamic magnetic resonance imaging (MRI), and abdominal arteriography. The diagnosis of HCC was made from a typical hypervascular tumor stain on angiography and a typical dynamic-study finding of enhanced staining in the early phase and attenuation in the delayed phase.

Treatment, follow-up, and determination of recurrence. Fifteen patients were treated with surgical resection, 41 with RFA, 19 with transarterial chemoembolization (TACE), and 10 with transarterial infusion (TAI). Among them, we selected 33 curative cases that met the following criteria: tumor stage classified as I or II; and surgical resection or RFA conducted for the initial HCC treatment. In all 33 cases, therapeutic effects were judged to be

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Table 1. Baseline demographic and clinical characteristics

Variable	Total patients (n = 85)
Sex (male/female)	54/31
Age (years)	73 [36–87]
BMI (kg/m^2)	23.2 [17.5–30.7]
Total body fat (cm^2)	188.81 [12.93–501.8]
Amount of visceral fat (cm^2)	76.43 [3.82–359.83]
Amount of subcutaneous fat (cm^2)	105.66 [9.11–265.26]
Etiology (B/C/B + C/other)*	8/55/2/20
Child-Pugh classification (A/B/C)	60/23/2
Ascites on CT imaging (present/absent)	7/78
ALB (g/dL)	3.5 [2.2–4.5]
PLT (×10^4/μL)	11.7 [3.0–76.4]
FPG (mg/dL)	97 [67–271]
FIRI (μU/mL)	8.115 [1.21–90.2]
HOMA-IR	2.245 [0.27–28.28]
HbA_{1c} (%)	5.3 [3.7–10.3]
Leptin (ng/mL)	5.0 [1.4–26.6]
Stage (I/II/III/IVA/IVB)	19/26/27/10/3
Initial treatment for HCC (resection/RFA/TACE/TAI)	15/41/19/10
AFP (ng/mL)	48 [0–222000]
PIVKA-II (mAU/mL)	170 [7–474000]
Follow-up period (days)	484 [14–1429]

Values are median [range]. *B means positive for hepatitis B surface antigen and C means positive for hepatitis C virus antibody. AFP, α-fetoprotein; B, hepatitis B virus; BMI, body mass index; C, hepatitis C virus; FPG, fasting plasma glucose; FIRI, fasting immunoreactive insulin; HbA_{1c}, hemoglobin A_{1c}; HCC, hepatocellular carcinoma; HOMA-IR, homeostasis model assessment of insulin resistance; PIVKA-II, protein induced by vitamin K absence or antagonists-II; RFA, radiofrequency ablation; TACE, transarterial chemoembolization; TAI, transarterial infusion.

curative using dynamic CT or MRI exhibiting a complete disappearance of the imaging characteristics of HCC described above.

Patients were thereafter followed up on a monthly outpatient basis using serum tumor markers every month, such as AFP and proteins induced by vitamin K absence or antagonists-II (PIVKA-II), and by abdominal US, dynamic CT scanning, or dynamic MRI every 3 months. Recurrent HCC was diagnosed, using the imaging modalities described earlier, by the appearance of other lesions differed from the primary lesions. The follow-up period was defined as the interval from the date of initial treatment until the date of diagnosis of recurrence or until April 2009 if HCC did not recur.

Statistical analysis. The Pearson product-moment correlation coefficient was used for measuring the linear correlation between 2 continuous variables. Recurrence-free survival was estimated using the Kaplan-Meier method, and differences between curves were examined with a log-rank test. Baseline characteristics were compared using Student's t test for continuous variables or the χ^2 test for categorical variables. There were 17 possible predictors for the recurrence of HCC after the initial curative treatment: sex, age, body mass index (BMI), total body fat, amounts of both visceral and subcutaneous fat, the presence of HCV-antibodies (HCV-Ab), Child-Pugh classification, serum albumin concentration, platelet count, homeostasis model assessment of insulin resistance (HOMA-IR = fasting plasma glucose (mg/dL) × fasting immunoreactive insulin (μU/mL)/405), hemoglobin A_{1c} (HbA_{1c}), serum tumor markers (AFP and PIVKA-II), initial treatment for HCC, tumor stage, and serum leptin concentration. Parameters that were significant as determined by univariate analysis were then subjected to multivariate analyses using the Cox proportional hazards model. Statistical significance was defined as $p<0.05$.

Results

Baseline characteristics and laboratory data of patients. The baseline characteristics and laboratory data of 85 patients (54 men and 31 women, median age 73 years) are shown in Table 1. The median follow-up period was 484 days (range, 14–1,429 days). Median BMI was 23.2 kg/m^2, which was classified in the normal range according to the WHO classification of obesity (http://www.who.int/bmi). Median free plasma glucose (FPG), free immunoreactive insulin (FIRI), HOMA-IR, and HbA_{1c} were 97 mg/dL, 8.115 μU/mL, 2.245, and 5.3%, respectively. The median serum leptin concentration was 5.0 ng/mL (range 1.4–26.6).

Association of the serum leptin concentration with obesity and insulin resistance. Four obesity-related factors were tested for possible association with the serum leptin concentration: BMI, total body fat, and the amounts of visceral and subcutaneous fat (Fig. 1). For BMI analysis, we excluded 7 patients with CT-detected ascites. The Pearson product-moment correlation coefficient and p values of BMI and the total body fat with serum leptin concentration were $r = 0.4559$ and $p<0.0001$, and $r = 0.3560$ and $p = 0.0008$, respectively; indicating that these 2 factors were significantly correlated with the serum leptin concentration. The amount of subcutaneous fat ($r = 0.5174$ and $p<0.0001$) was also strongly correlated with the serum leptin level, whereas the amount of visceral fat ($r = 0.0987$ and $p = 0.3776$) was not. In addition, no significant correlations were noted between the serum leptin concentration and insulin resistance-related factors, including FPG ($r = -0.0816$ and $p = 0.4579$), FIRI ($r = 0.1049$ and $p = 0.3378$), HOMA-IR ($r = 0.0506$ and $p = 0.6385$), and HbA_{1c} ($r = 0.0194$ and $p = 0.7820$).

Possible risk factors for the recurrence of HCC. In all 33 curative cases of stage I/II HCC, 12 patients experienced recur-

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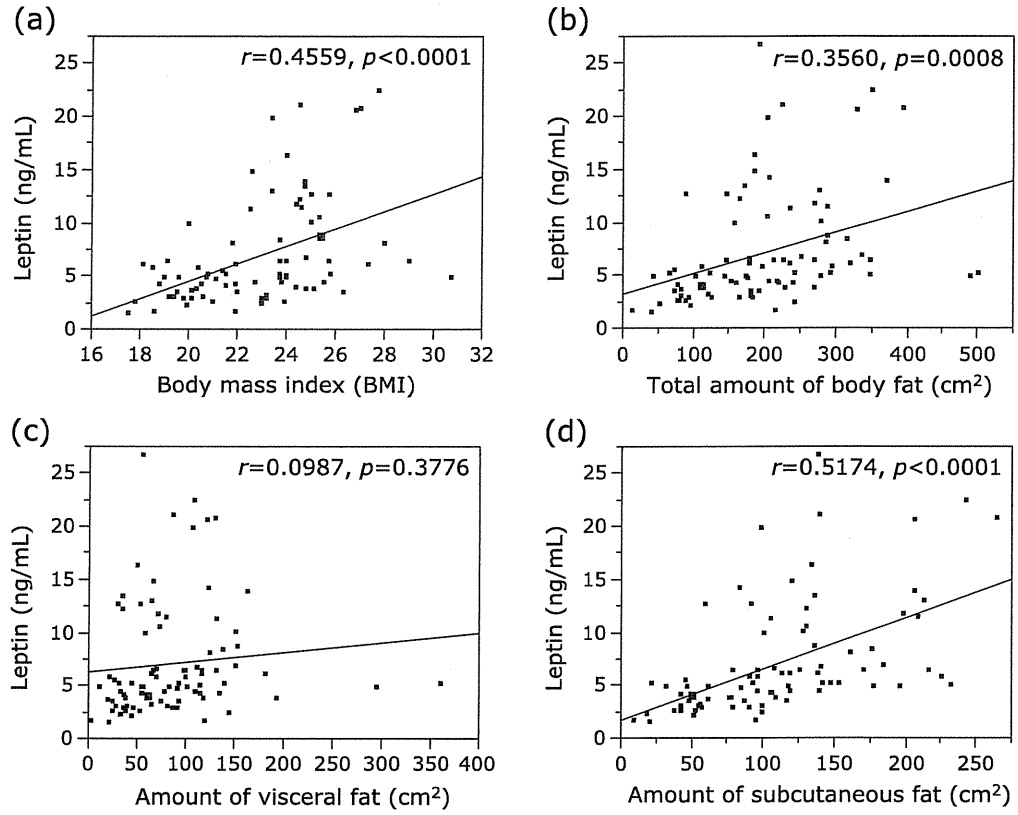

Fig. 1. Correlation between the serum levels of leptin and (a) BMI, (b) total body fat, (c) amount of visceral fat, and (d) amount of subcutaneous fat in patients with HCC (n = 85). For BMI analysis, we excluded 7 patients with CT-detected ascites.

rence in the liver, but none exhibited distant metastasis. The 1-year recurrence-free survival in the 33 patients was 79% (Fig. 2a). Fig. 2b shows Kaplan-Meier curves for recurrence-free survival divided into 2 subgroups on the basis of median serum leptin concentration (≤5 or >5 ng/mL), which results in a significant difference (p = 0.0221).

The Cox proportional hazards model was used to analyze risk factors for the recurrence of stage I/II HCC after curative treatments using the 17 variables listed in Table 2. BMI (hazard ratio 1.30, 95% CI 1.08–1.56, p = 0.0062), total body fat (hazard ratio 1.00, 95% CI 1.00–1.01, p = 0.0404), serum albumin concentration (hazard ratio 0.26, 95% CI 0.08–0.81, p = 0.0210), AFP (hazard ratio 0.99, 95% CI 0.99–0.99, p = 0.0365), and serum leptin concentration (hazard ratio 1.29, 95% CI 1.12–1.50, p = 0.0003) were identified as significant risk factors by univariate analysis. Multivariate analysis only identified serum leptin concentration (hazard ratio 1.25, 95% CI 1.07–1.49, p = 0.0035) as significant independent risk factor for the recurrence of HCC (Table 3).

Table 4 shows the baseline characteristics and laboratory data of patients divided on the basis of the serum leptin concentration (≤5 and >5 ng/mL). No significant differences were noted between the 2 subgroups, except the amount of subcutaneous fat (p = 0.0461).

Discussion

Leptin regulates body weight by signaling information to the brain regarding the availability of energy stored as fat; this negative feedback loop is disrupted in most obese individuals and results in a state known as leptin resistance.[16,24] Consistent with the results of previous studies,[16,24] the serum leptin concentration was significantly correlated with BMI and total body fat in the present study (Fig. 1 a and b). These parameters were also significant risk factors for the recurrence of HCC as determined by univariate analysis (Table 2); however, the serum leptin concentration was the most significant biomarker (p = 0.0003).

In addition, we clearly showed for the first time that patients with greater serum leptin concentrations were susceptible to HCC recurrence (Fig. 2b); thus, increased serum leptin levels are a significant independent risk factor for the recurrence of this malignancy (Table 3). This finding indicates that increased serum leptin concentration, which might link obesity with liver carcinogenesis, is a preferable and useful biomarker for screening high-risk groups for the recurrence of HCC. We previously reported that a state of insulin resistance associated with obesity is an independent risk factor for the recurrence of HCC after curative treatment.[10] Furthermore, no significant correlations between serum leptin levels and insulin resistance-related factors were noted in the present study, suggesting these two conditions might be independent from each other in HCC patients. Therefore, a combination evaluation for both the serum leptin level and insulin resistance would be more effective for screening high-risk groups for HCC, and requires future confirmation.

Several studies report that leptin is a risk factor for carcinogenesis at various organ sites, including the liver.[17–21] Leptin can stimulate cellular proliferation in various types of cancer cells such as HCC cells.[19–21,25] In addition, when focusing on the liver,

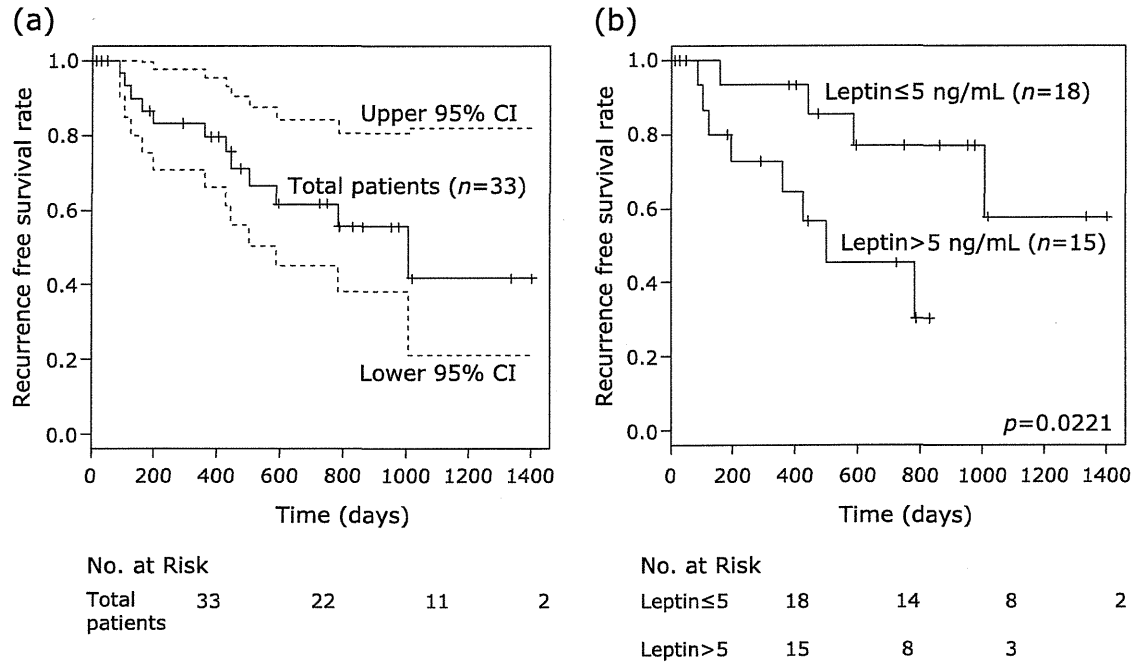

Fig. 2. Kaplan-Meier curves for recurrence-free survival in (a) total patients and in (b) subgroups divided on the basis of the serum leptin concentration (≤5 or >5 ng/mL).

Table 2. Univariate analyses of possible risk factors for recurrence-free survival of HCC using the Cox proportional hazards model

Variable	95% CI			
	HR*	lower	upper	*p* value
Sex (male vs female)	0.9	0.28	3.09	0.8726
Age (years)	0.96	0.89	1.03	0.277
BMI (kg/m^2)	1.3	1.08	1.56	0.0062
Total body fat (cm^2)	1	1	1.01	0.0404
Amount of visceral fat (cm^2)	1	0.99	1.01	0.0909
Amount of subcutaneous fat (cm^2)	1	0.99	1.01	0.0601
The presence of HCV-Ab (yes vs no)	0.42	0.12	1.98	0.2501
Child-Pugh classification (B + C vs A)	1.33	0.35	4.3	0.6482
ALB (g/dL)	0.26	0.08	0.81	0.021
PLT (×10^4/μL)	0.87	0.75	1.01	0.0714
HOMA-IR	1.03	0.94	1.1	0.4
HbA_{1c} (%)	0.87	0.37	1.6	0.7108
AFP (ng/mL)	0.99	0.99	0.99	0.0365
PIVKA-II (mAU/mL)	0.99	0.99	1	0.7448
Initial treatment for HCC (RFA vs resection)	1.61	0.42	10.5	0.5128
Stage (II vs I)	1.08	0.32	3.78	0.89
Leptin (ng/mL)	1.29	1.12	1.5	0.0003

*HR represents the values with a unit increase in continuous variables. AFP, α-fetoprotein; BMI, body mass index; CI, confidence interval; HbA_{1c}, hemoglobin A_{1c}; HCC, hepatocellular carcinoma; HCV-Ab, hepatitis C virus antibody; HOMA-IR, homeostasis model assessment of insulin resistance; HR, hazard ratio; PIVKA-II, protein induced by vitamin K absence or antagonists-II; RFA, radiofrequency ablation.

leptin is a potent profibrogenic cytokine and thus plays a key role in the progression of cirrhosis,[26] which is a precancerous condition of HCC. Indeed, increased serum leptin concentration has been documented in cirrhotic patients.[27,28] Moreover, increased leptin expression is associated with increased intratumor microvascular density. Consequently, it is hypothesized that leptin plays a stimulatory role in the development of HCC via neovascularization.[29] In addition to using leptin as a biomarker for the risk of HCC recurrence, the present findings suggest that targeting leptin might be an effective strategy for the prevention and treatment of

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Table 3. Multivariate analyses of possible risk factors for recurrence-free survival of HCC using the Cox proportional hazards model

Variable	95% CI			
	HR*	lower	upper	*p* value
BMI (kg/m^2)	1.2	0.83	1.81	0.3278
Total body fat (cm^2)	1	0.99	1.01	0.8003
ALB (g/dL)	0.54	0.12	2.28	0.4018
AFP (ng/mL)	0.99	0.99	1	0.1416
Leptin (ng/mL)	1.25	1.07	1.49	0.0035

*HR represents the values with a unit increase in continuous variables. AFP, α-fetoprotein; BMI, body mass index; CI, confidence interval; HR, hazard ratio.

Table 4. Baseline demographic and clinical characteristics of patients classified on the basis of the serum leptin concentration

Variable	Leptin ≤ 5 ng/mL (*n* = 18)	Leptin > 5 ng/mL (*n* = 15)	*p* value
Sex (male/female)	13/5	6/9	0.0604
Age (years)	72.5 [59–87]	70 [50–85]	0.2565
BMI (kg/m^2)	21.5 [17.8–30.7]	24.5 [18.5–27.7]	0.1111
Total body fat (cm^2)	167.5 [73.9–490.9]	207.3 [112.2–350.8]	0.2591
Amount of visceral fat (cm^2)	69.4 [19.9–294.4]	98.9 [21.9–181.6]	0.9479
Amount of subcutaneous fat (cm^2)	90.2 [42.0–232.3]	134.3 [79.6–242.5]	0.0461
Etiology (C/others)	14/4	11/4	0.767
Child-Pugh classification (A/B/C)	15/3/0	10/5/0	0.2657
ALB (g/dL)	3.6 [2.6–4.2]	3.3 [2.4–4.4]	0.2708
PLT (×10^4/μL)	12.45 [7.7–26.1]	9.5 [3.0–20.6]	0.0895
FPG (mg/dL)	97.5 [83–271]	105 [75–154]	0.7424
FIRI (μU/mL)	6.05 [2.57–65.2]	14.3 [7.3–27.4]	0.3657
HOMA-IR	1.51 [0.53–24.8]	3.41 [1.45–9.40]	0.641
HbA_{1c} (%)	5.3 [4.5–10.3]	5.2 [3.7–6.8]	0.3351
Stage (I/II)	7/11	9/6	0.2253
Initial treatment for HCC (resection/RFA)	6/12	2/13	0.1726
AFP (ng/mL)	8 [0–20500]	28 [1–2530]	0.1687
PIVKA-II (mAU/mL)	22.7 [8–201000]	26 [7–29800]	0.4385

Values are median [range]. AFP, α-fetoprotein; C, hepatitis C virus; FPG, fasting plasma glucose; FIRI, fasting immunoreactive insulin; HbA_{1c}, hemoglobin A_{1c}; HCC, hepatocellular carcinoma; HOMA-IR, homeostasis model assessment of insulin resistance; PIVKA-II, protein induced by vitamin K absence or antagonists-II; RFA, radiofrequency ablation.

HCC. Ribatti *et al.* state that anti-leptin antibodies reduce the angiogenic response in HCC biopsy specimens.[29] Decreases in serum leptin are also associated with the prevention of obesity-related liver tumorigenesis in obese and diabetic mice models.[14]

In conclusion, we report that patients with high serum leptin concentrations are susceptible to HCC recurrence in stage I/II cases curatively treated by surgical resection or RFA. Increased serum leptin concentration may be a useful biomarker for predicting the recurrence of HCC in high-risk patients.

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Abbreviations

AFP	α-fetoprotein
BMI	body mass index
CT	computed tomography
DM	diabetes mellitus
FPG	fasting plasma glucose
FIRI	fasting immunoreactive insulin
HbA_{1c}	hemoglobin A_{1c}
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HOMA-IR	homeostasis model assessment of insulin resistance
LCSGJ	Liver Cancer Study Group of Japan
MRI	magnetic resonance imaging
PIVKA-II	protein induced by vitamin K absence or antagonists-II
RFA	radiofrequency ablation
TACE	transarterial chemoembolization
TAI	transarterial infusion
US	ultrasonography

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Renin–angiotensin system inhibitors suppress azoxymethane-induced colonic preneoplastic lesions in C57BL/KsJ-*db/db* obese mice

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Oxidative stress

ABSTRACT

Obesity-related metabolic abnormalities, including chronic inflammation and oxidative stress, increase the risk of colorectal cancer. Dysregulation of the renin–angiotensin system (RAS) also plays a critical role in obesity-related metabolic disorders and in several types of carcinogenesis. In the present study, we examined the effects of an angiotensin-converting enzyme (ACE) inhibitor and angiotensin-II type 1 receptor blocker (ARB), both of which inhibit the RAS, on the development of azoxymethane (AOM)-initiated colonic premalignant lesions in C57BL/KsJ-*db/db* (*db/db*) obese mice. Male *db/db* mice were given 4 weekly subcutaneous injections of AOM (15 mg/kg body weight), and then, they received drinking water containing captopril (ACE inhibitor, 5 mg/kg/day) or telmisartan (ARB, 5 mg/kg/day) for 7 weeks. At sacrifice, administration of either captopril or telmisartan significantly reduced the total number of colonic premalignant lesions, i.e., aberrant crypt foci and β-catenin accumulated crypts, compared to that observed in the control group. The expression levels of TNF-α mRNA in the colonic mucosa of AOM-treated *db/db* mice were decreased by captopril and telmisartan. Captopril lowered the expression levels of TNF-α, IL-1β, IL-6, and PAI-1 mRNAs, while telmisartan lowered the expression levels of COX-2, IL-1β, IL-6, and PAI-1 mRNAs in the white adipose tissues of these mice. In addition, these agents significantly reduced the levels of urinary 8-OHdG, a surrogate marker of oxidative damage to DNA, in the experimental mice. These findings suggested that both ACE inhibitor and ARB suppress chemically-induced colon carcinogenesis by attenuating chronic inflammation and reducing oxidative stress in obese mice. Therefore, targeting dysregulation of the RAS might be an effective strategy for chemoprevention of colorectal carcinogenesis in obese individuals.

1. Introduction

Mounting evidence indicates that obesity, a result of a positive energy balance, and its related metabolic abnormalities raise the risk of colorectal cancer (CRC) [1,2]. Obesity is regarded as a state of chronic inflammation, which is closely associated with colorectal carcinogenesis [3]. Increased levels of adipose tissue lead to the expression of a variety of pro-inflammatory cytokines, including tumor necrosis factor-α (TNF-α) [4], which stimulates tumor promotion and progression of carcinogenesis [5]. Oxidative stress, which is induced by increased energy availability [6], has also been suggested to play an important role in the development of CRC [1,2]. Thus, these findings suggest that targeting inflammation and oxidative stress may be an effective strategy for preventing the development of CRC, especially in overweight individuals. For instance, a recent study shows that administration of pitavastatin, a hypolipidemic drug, prevents obesity-related colorectal tumorigenesis by attenuating chronic inflammation [7].

Hyperactivity of the renin–angiotensin system (RAS), an endocrine system with critical roles in cardiovascular function, has been implicated in the etiology of high blood pressure, obesity, and metabolic syndrome [8]. In addition, there is strong evidence that the RAS is frequently dysregulated in human malignancies, which correlates with poor patient outcomes. Abnormalities in the RAS influences cancer cell migration, invasion, and metastasis, all of which are closely associated with chronic inflammation and angiogenesis [9,10]. In cancer tissues, the RAS is upregulated through systemic

Abbreviations: ACE, angiotensin converting enzyme; ACF, aberrant crypt foci; AOM, azoxymethane; ARB, angiotensin-II type-1 receptor blocker; BCAC, β-catenin accumulated crypt; COX-2, cyclooxygenase-2; CRC, colorectal cancer; *db/db* mice, C57BL/KsJ-*db/db* mice; ELISA, enzyme-linked immunosorbent assay; H&E, hematoxylin and eosin; IL, interleukin; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; PAI-1, plasminogen activator inhibitor-1; RAS, renin–angiotensin system; RT-PCR, reverse transcription-PCR; TNF-α, tumor necrosis factor-α.

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oxidative stress and hypoxia mechanisms, which triggers chronic inflammatory processes to remodel the surrounding environment [11].

Drugs that reduce the synthesis (angiotensin-converting enzyme [ACE] inhibitors) or action (angiotensin-II type-1 receptor blockers [ARBs]) of angiotensin-II, the active product of RAS, are widely used for the treatment of hypertension. These agents have also been expected to exert beneficial effects that improve the symptoms of metabolic disorders [12,13]. In addition, retrospective studies have shown that patients taking ACE inhibitors or ARBs had decreased risk of developing some types of cancers, including CRC [14–16]. The expression levels of ACE are higher in colorectal adenomas and CRC epithelial cells than in the corresponding non-neoplastic crypt and surface epithelia [17]. In a mouse model of CRC liver metastasis, administration of an ACE inhibitor and ARB significantly reduced tumor volume by blocking the RAS activity [18]. These reports suggest that the RAS might be a critical target for the treatment and/or prevention of certain types of human malignancies, including CRC. However, the possibility of CRC chemoprevention by targeting the RAS is yet to be considered.

The C57BL/KsJ-*db/db* (*db/db*) mouse is one of the most widely used models of type 2 diabetes. The development of diabetes in *db/db* mice results in the activation of RAS and induction of oxidative stress, which promotes progressive inflammation [19]. In the present study, we used male *db/db* mice injected with azoxymethane (AOM) to examine the effects of captopril (ACE inhibitor) and telmisartan (ARB) on the development of aberrant crypt foci (ACF) and β-catenin accumulated crypts (BCAC), both of which are putative precursor lesions for colonic adenocarcinoma [20,21], by focusing on the attenuation of inflammation and reduction of oxidative stress. This preclinical animal model is useful for investigating specific agents for their ability to prevent inflammation-related colorectal carcinogenesis caused by obesity [7].

2. Materials and methods

2.1. Animals, chemicals, and diet

Male homozygous *db/db* mice aged 4 weeks (Japan SLC, Inc., Shizuoka, Japan) were maintained at the Gifu University Life Science Research Center in accordance with the Institutional Animal Care Guidelines. AOM, captopril, and telmisartan were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Experimental procedure

The animal experiment, as described previously [7,22,23], was approved by the Committee of the Institutional Animal Experiments of Gifu University. A total of 45 male *db/db* mice were divided into six groups. To induce colonic preneoplastic lesions, at 5 weeks of age, the mice in Groups 4 (10 mice), 5 (10 mice), and 6 (10 mice) were given 4 weekly subcutaneous injections of AOM (15 mg/kg body weight). The mice in Groups 1 (5 mice), 2 (5 mice), and 3 (5 mice) were subcutaneously injected with saline once a week for 4 weeks. Groups 2 and 5 received drinking water containing captopril (5 mg/kg/day) for 7 weeks, starting 1 week after the last injection of AOM. Similarly, the mice in Groups 3 and 6 were given drinking water containing telmisartan (5 mg/kg/day). Captopril and telmisartan intake was maintained by adjusting the concentration of these agents in drinking water, whose volume was measured three times a week. Groups 1 and 4 were given tap water throughout the experiment. At the end of the study (16 weeks of age), all the mice were sacrificed by CO_2 asphyxiation for colon resection. The third portion of excised colons (cecum side) was used to extract RNA, and the remaining part was used to determine the numbers of colonic ACF and BCAC.

2.3. Counting the number of ACF and BCAC

The frequency of ACF and BCAC was determined according to the standard procedures [7,22,23]. The colon samples fixed with 10% buffered formalin were stained with methylene blue (0.5% in distilled water), and the number of ACF was counted under a light microscope. To identify BCAC intramucosal lesions, the distal part (1 cm from the anus) of the colon (mean area: 0.7 cm^2/colon) was embedded in paraffin, and 20 serial sections (4-μm thick) per mouse were created by an *en face* preparation. The sections were then subjected to H&E staining for histopathology and β-catenin immunohistochemistry to count the number of BCAC. The anti-β-catenin primary antibody was purchased from BD Transduction Laboratories (San Jose, CA, USA), and immunohistochemical staining was performed using a labeled streptavidin–biotin method (DAKO, Glostrup, Denmark). β-Catenin-stained BCACs were counted and the values were expressed as per cm^2 of mucosa [7,22,23].

2.4. RNA extraction and quantitative real-time reverse transcription-PCR

The expression levels of TNF-α and interleukin (IL)-6 genes in the colonic mucosa and those of TNF-α, cyclooxygenase (COX)-2, IL-1β, IL-6, and plasminogen activator inhibitor-1 (PAI-1) genes in the white adipose tissues of AOM-treated *db/db* mice were determined by quantitative real-time reverse transcription-PCR (RT-PCR) analysis [7,24]. Total RNA was isolated using the RNAqueous-4PCR Kit (Applied Biosystems, Austin, TX, USA). cDNA was synthesized from 0.2 μg of total RNA by using the SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). The specific primers used for the amplification of TNF-α, COX-2, IL-1β, IL-6, and β-actin genes were as previously described [24]. The specific primers used for amplification of the PAI-1 gene were as follows: sense 5′-TTC AGC CCT TGC TTG CCT C-3′ and antisense 5′-ACA CTT TTA CTC CGA AGT CGG T-3′. Real-time RT-PCR was performed using a LightCycler (Roche Diagnostics GmbH, Mannheim, Germany) with the SYBR Premix Ex Taq (TaKaRa Bio Inc., Shiga, Japan). The expression level of each gene was normalized to that of the β-actin gene by using the standard curve method.

2.5. Measurement of urinary 8-OHdG levels

Urine samples were collected at the time of sacrifice, and the levels of 8-hydroxy-2′-deoxyguanosine (8-OHdG) were determined by using an enzyme-linked immunosorbent assay (ELISA) kit (NIKKEN SEIL, Shizuoka, Japan) according to the manufacturer's protocol.

2.6. Statistical analyses

The statistical analyses were performed using the JMP 8 software program (SAS Institute, Cary, NC, USA), and the results are presented as mean (SD). Statistical significance was evaluated using Dunnet's *t*-test for multiple comparisons. Differences were considered statistically significant when the two-tailed *p*-value was less than 0.05.

3. Results

3.1. General observations

As listed in Table 1, the average body weight and relative liver weight of the AOM-injected groups (Groups 4–6) at the end of the experiment were significantly ($p < 0.01$) lower than those of

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

Group No.	Treatment	No. of mice	Body weight (g)	Relative weight (g/100 g body weight) of:	
				Liver	Kidney
1	Saline	5	54.6 ± 8.8[a]	5.95 ± 0.92	0.90 ± 0.26
2	Saline + captopril	5	58.2 ± 1.8	6.28 ± 0.44	0.79 ± 0.07
3	Saline + telmisartan	5	62.0 ± 3.6	7.65 ± 1.05	0.76 ± 0.10
4	AOM alone	10	40.9 ± 5.5[b]	4.65 ± 0.60[b]	0.93 ± 0.27
5	AOM + captopril	10	37.4 ± 8.4[b]	4.53 ± 0.64[b]	1.05 ± 0.22
6	AOM + telmisartan	10	42.2 ± 8.8[b]	4.49 ± 0.55[b]	0.96 ± 0.15

[a] Mean ± SD.
[b] Significantly different from Group 1 ($p < 0.01$).

the saline-injected group (Group 1). This might be caused by the toxicity of AOM, as observed in previous experiments [7,22,23]. No significant differences were observed in the mean relative weight of the kidney among the groups. No histopathological findings suggesting toxicity of captopril or telmisartan in the liver, kidney, and spleen of the mice were obtained (data not shown).

3.2. Effects of captopril and telmisartan on AOM-induced ACF and BCAC in db/db mice

Table 2 summarizes the total number of ACF (Fig. 1A and B) and BCAC (Fig. 1C and D) in the mice from all groups. Both ACF and BCAC developed in the colons of all mice that received AOM (Groups 4–6), but not in those without AOM treatment (Groups 1–3). When compared with Group 4 (AOM alone), administration of either captopril or telmisartan in drinking water significantly reduced ACF frequency; the inhibition rates were 43% in Group 5 (AOM + captopril, $p < 0.01$) and 39% in Group 6 (AOM + telmisartan, $p < 0.01$). Similarly, both captopril- (76% reduction, $p < 0.01$) and telmisartan- (71% reduction, $p < 0.01$) treatment groups had significantly decreased numbers of BCAC than the AOM alone-treated group.

3.3. Effects of captopril and telmisartan on the expression levels of TNF-α and IL-6 mRNA in the colonic mucosa of AOM-treated db/db mice

TNF-α is an important tumor promoter involved in obesity, inflammation, and carcinogenesis [3–5]. As shown in Fig. 2A, quantitative real-time RT-PCR analyses showed that both captopril and telmisartan significantly decreased the expression levels of TNF-α mRNA in the colonic mucosa of AOM-treated mice ($p < 0.05$). On the other hand, the expression levels of IL-6 mRNA in the colonic mucosa (Fig. 2B), which also are possibly involved in obesity- and inflammation-related colorectal carcinogenesis [3,25], were not significantly lowered by treatment with these agents.

3.4. Effects of captopril and telmisartan on the expression levels of TNF-α, COX-2, IL-1β, IL-6, and PAI-1 mRNA in the white adipose tissues of AOM-treated db/db mice

In the white adipose tissues of AOM-treated *db/db* mice, the expression levels of TNF-α (Fig. 3A), IL-1β (Fig. 3C), IL-6 (Fig. 3D), and PAI-1 (Fig. 3E) mRNAs were significantly inhibited by captopril administration compared to the control mice ($p < 0.05$ for each). Drinking telmisartan also caused a decrease in the expression levels of COX-2 (Fig. 3B), IL-1β (Fig. 3C), IL-6 (Fig. 3D), and PAI-1 (Fig. 3E) mRNAs in the white adipose tissues of AOM-treated mice ($p < 0.05$). These findings (Figs. 2 and 3) indicated that administration of these agents attenuates the inflammatory response in the colonic mucosa and in the white adipose tissues of obese mice.

3.5. Effects of captopril and telmisartan on the urinary levels of 8-OHdG in AOM-treated db/db mice

Urinary 8-OHdG levels in AOM-treated *db/db* mice were determined using ELISA method (Fig. 4). The mice treated with either captopril (6.5 ± 2.0 ng/mL) or telmisartan (7.3 ± 2.2 ng/mL) showed a significant decrease in the urinary levels of 8-OHdG compared to the untreated mice (17.9 ± 3.5 ng/mL; $p < 0.01$ for each comparison). These findings indicated that captopril and telmisartan suppresses obesity-related systemic oxidative stress.

4. Discussion

There is accumulating evidence to indicate that abnormalities in the RAS play a critical role in several types of carcinogenesis; therefore, agents targeting the RAS might augment cancer therapies [9,10]. The results of the present study clearly indicated that the RAS inhibitors captopril and telmisartan effectively suppress the development of colonic preneoplastic lesions, ACF and BCAC, in male *db/db* obese mice. This is the first report that shows the preventive effect of an ACE inhibitor and ARB on the development of chemically-induced colorectal carcinogenesis in any mouse

Table 2
Effects of captopril and telmisartan on AOM-induced ACF and BCAC formation in the experimental mice.

Group No.	Treatment	No. of mice	Length of colon (cm)	Total No. of ACFs/colon	Total No. of BCACs/cm^2
1	Saline	5	11.1 ± 0.8 [a]	0	0
2	Saline + captopril	5	11.9 ± 0.9	0	0
3	Saline + telmisartan	5	11.7 ± 0.8	0	0
4	AOM alone	10	10.6 ± 0.8	134.0 ± 24.5	3.4 ± 1.8
5	AOM + captopril	10	10.6 ± 1.3	76.9 ± 24.3[b]	0.8 ± 0.9[b]
6	AOM + telmisartan	10	10.8 ± 0.7	81.8 ± 14.0[b]	1.0 ± 1.0[b]

[a] Mean ± SD.
[b] Significantly different from Group 4 ($p < 0.01$).

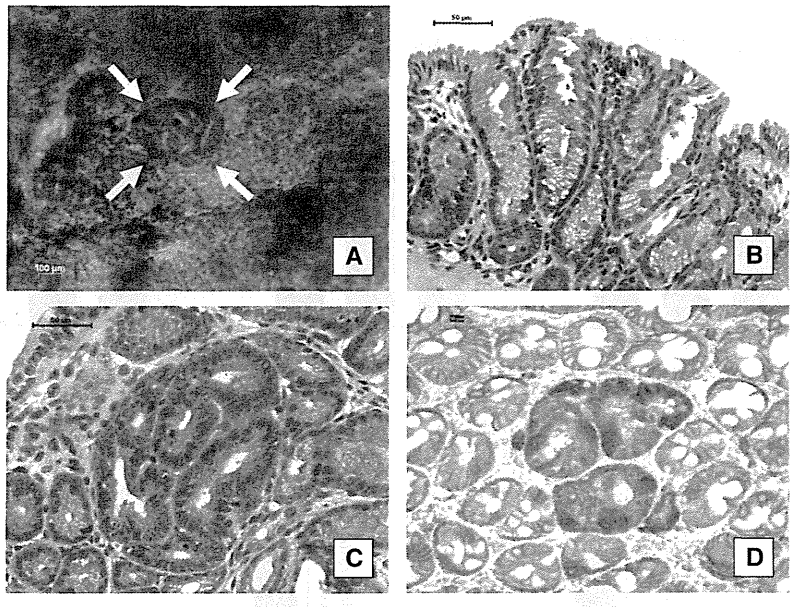

Fig. 1. Histopathology and β-catenin-immunohistochemistry of ACF and BCAC in AOM-exposed *db/db* mice (Group 4). Arrows indicate ACF (A) stained by methylene blue on the colonic mucosa. Representative photographs of ACF (B) and BCAC (C) stained with H&E. Basophilic cytoplasm and hyperchromatic nuclei are observed in the atypical cryptal cells in BCAC (C). Immunohistochemistry of β-catenin protein in BCAC (D). The localization of the accumulated β-catenin protein is apparent in the cytoplasm and nucleus of atypical cryptal cells. Scale bars, 100 μm (A) and 50 μm (B–D).

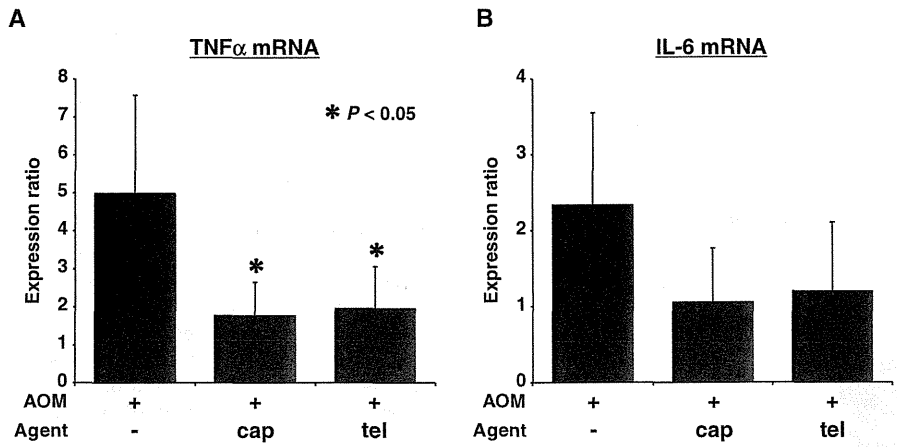

Fig. 2. The effects of captopril and telmisartan on the expression levels of TNF-α and IL-6 mRNAs in the colonic mucosa of AOM-treated *db/db* mice. cDNA was synthesized from scraped colonic mucosa, and real-time RT-PCR was performed using specific primers for TNF-α (A) and IL-6 (B). The expression levels of these genes were normalized to that of the β-actin gene. Data represent mean ± SD ($n = 8$). $^*p < 0.05$ vs. AOM-treated control group.

model. The finding seemed to be significant in clinical medicine because these drugs are widely used for patients with hypertension who frequently are obese. Furthermore, high blood pressure is involved in the increased risk of development of CRC and colonic adenomas [26–28], thus indicating that obese and hypertensive patients might be regarded as a high-risk group for CRC development. On the other hand, a recent retrospective study shows that use of an ACE inhibitor is significantly associated with reduction in the incidence and size of colorectal adenomas, the precancerous lesions for CRC [29]. This report [29] along with the results of the present study suggests that inhibition of RAS might be an effective strategy for the prevention of colorectal tumorigenesis, especially in obese individuals.

A key feature of obesity is increased inflammation in the adipose tissue, which might be involved in cancer promotion and progression [3]. Angiotensin-II is considered a pro-inflammatory mediator because activation of its receptor induces a number of molecules that participate in inflammatory responses [8–10]. For instance, mice with elevated adipocyte angiotensinogen expression have increased the expression of TNF-α, IL-6, and IL-1β in the adipose tissue [30]. Treatment with ARB decreased plasma levels of TNF-α and IL-6 in patients with congestive heart failure [31]. In the present study, either captopril or telmisartan decreased the mRNA levels of TNF-α, COX-2, IL-1β, IL-6, and PAI-1 in the white adipose tissue of AOM-treated *db/db* mice. Therefore, the chemopreventive effect of an ACE inhibitor and ARB on obesity-related colorectal carcinogenesis is most likely associated with the attenuation of systemic inflammation. In addition, the inhibition of the expression levels of TNF-α mRNA in the colonic mucosa might also contribute to this beneficial effect because this cytokine promotes

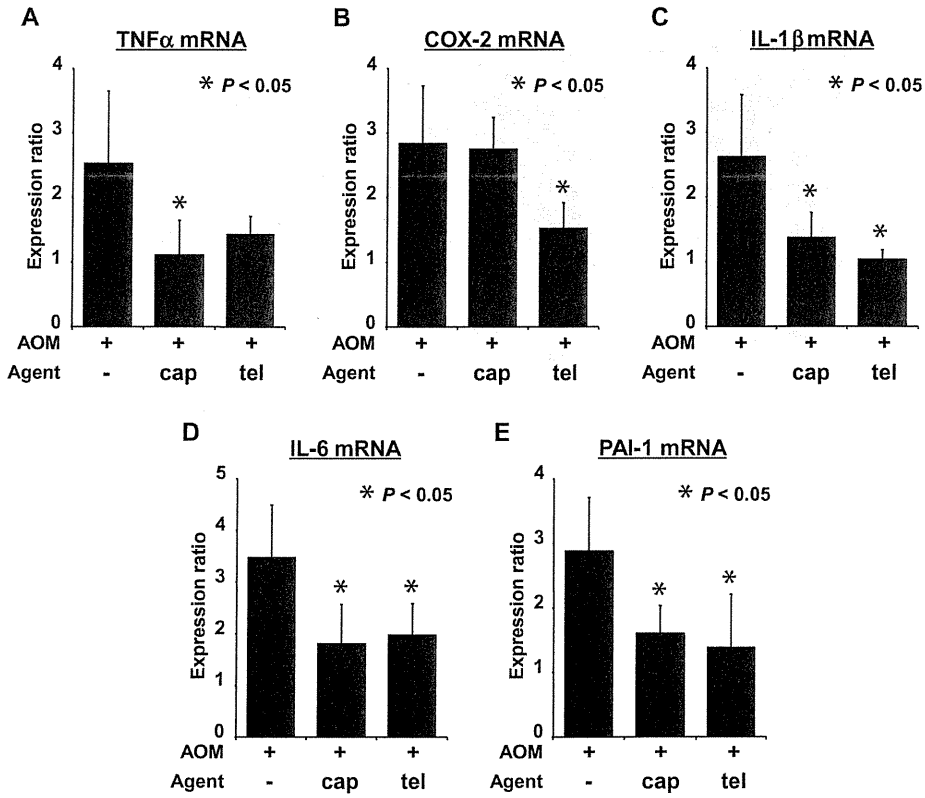

Fig. 3. The effects of captopril and telmisartan on the expression levels of TNF-α, COX-2, IL-1β, IL-6, and PAI-1 mRNAs in the white adipose tissues of AOM-treated *db/db* mice. cDNA was synthesized from the white adipose tissues of the retroperitoneum, and real-time RT-PCR was performed using specific primers for TNF-α (A), COX-2 (B), IL-1β (C), IL-6 (D), and PAI-1 (E). The expression levels of these genes were normalized to that of the β-actin gene. Data represent mean ± SD ($n = 8$). $^*p < 0.05$ vs. AOM-treated control group.

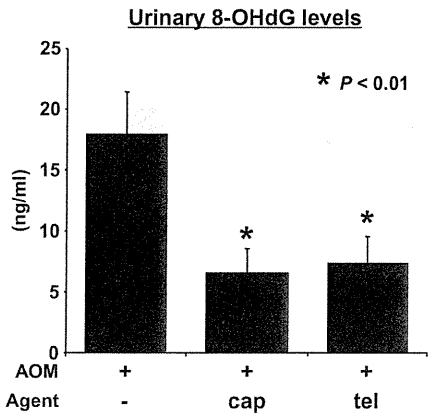

Fig. 4. The effects of captopril and telmisartan on urinary 8-OHdG levels in AOM-treated *db/db* mice. At sacrifice, urine samples were collected from the experimental mice, and the levels of urinary 8-OHdG were measured by ELISA. Data represent mean ± SD ($n = 8$). $^*p < 0.01$ vs. AOM-treated control group.

inflammation-related colorectal carcinogenesis, and thus, are critical targets for CRC chemoprevention [5,7,32].

Increased oxidative stress, which is associated with obesity due to metabolic and inflammatory changes [6], promotes damage to cell structures including DNA; this plays a key role in cancer development [33]. Certain types of chemopreventive agents, such as polyphenolic compounds, inhibit colorectal carcinogenesis by exerting anti-oxidant effects [34,35]. Activation of the RAS by enhanced levels of angiotensin-II leads to an increase in oxidative stress [36], but this is significantly reduced by treatment with RAS inhibitors [37,38]. In prostate cancer cells, candesartan, an ARB, also significantly reduces angiotensin-II-upregulated oxidative stress [39]. In the present study, both captopril and telmisartan decreased the levels of urinary 8-OHdG, which is a useful marker of DNA damage induced by oxidative stress, and this might be associated with inhibition of colorectal carcinogenesis. These findings, together with the results of recent studies [36–39], suggest that increased oxidative stress might be a critical target of RAS inhibitors for suppression of CRC.

Recent studies have revealed that insulin resistance and hyperinsulinemia, which are closely related to obesity, are some of the key factors in the development of obesity-related CRC, and thus, may be critical targets for the prevention of this malignancy [1,2,22,23]. In addition, activation of the RAS has been implicated in the etiology of obesity and insulin resistance [8,40]. Therefore, in the present study, it was expected that captopril and telmisartan might improve insulin resistance. However, contrary to our expectations, there was no clear evidence indicating an improvement in insulin resistance by these agents (data not shown). Therefore, at least in the present study, insulin resistance might not be a critical target of ACE inhibitors and ARBs to prevent colorectal tumorigenesis in obese mice.

The present experimental study was performed using *db/db* obese mice, which exhibit increased RAS activation and oxidative stress [19]. These mice are also highly susceptible to the colonic carcinogen AOM compared to the wild (+/+) and heterozygous

db/+ mice, neither of which exhibit obesity [41,42]. However, a question whether RAS inhibitors can prevent CRC development under non-obese condition has not yet been determined. Therefore, further studies that can clarify the effects of RAS inhibitors on the development of CRC under physiological RAS condition are required to confirm the possibility that these agents can be widely used as chemopreventive drugs for CRC.

In summary, prevention of CRC by targeting chronic inflammation and oxidative stress, which is caused by obesity and is related to RAS activation, might be a promising chemopreventive strategy for obese people, who are at an increased risk of developing CRC. Therefore, the agents targeting RAS, including ACE inhibitors and ARBs, appear to be potentially effective candidates for this purpose because these drugs attenuate inflammation while reducing oxidative stress.

Conflicts of interest

The authors declare that no conflicts of interest exist.

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Short Communication

Combination of acyclic retinoid with branched-chain amino acids inhibits xenograft growth of human hepatoma cells in nude mice

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Aim: Combination chemoprevention is a promising strategy to improve the prognosis of hepatocellular carcinoma (HCC). A malfunction of retinoid X receptor-α (RXR-α) due to phosphorylation by Ras/mitogen-activated protein kinase is closely associated with liver carcinogenesis and acyclic retinoid (ACR) can prevent HCC development by inhibiting RXR-α phosphorylation. The present study examined the possible combined effects of ACR plus branched-chain amino acids (BCAA), which can also prevent the development of HCC in obese patients with liver cirrhosis, in human HCC xenografts in nude mice.

Methods: This study examined the effects of the combination of ACR plus BCAA on the growth of Huh7 human HCC xenografts in nude mice. The effects of the combination on the phosphorylation of RXR-α, extracellular signal-regulated kinase (ERK), Akt and insulin-like growth factor-1 receptor (IGF-1R) proteins, and on the expression levels of retinoic acid receptor-β (RAR-β) and $p21^{CIP1}$ mRNA, were also examined by western blot and real-time reverse transcription polymerase chain reaction analyses, respectively.

Results: The combined treatment with ACR plus BCAA significantly inhibited the growth of Huh7 xenografts. The combination of these agents caused a marked inhibition of the phosphorylation of RXR-α, ERK, Akt and IGF-1R proteins in the xenografts. In addition, the expression levels of RAR-β and $p21^{CIP1}$ mRNA significantly increased by these agents.

Conclusion: The combination of ACR and BCAA restores the function of RXR-α by inhibiting its phosphorylation and increasing the level of RAR-β, a heterodimeric partner for RXR-α, and thus suppresses the growth of HCC xenografts. Therefore, this combination might be an effective regimen for the treatment and, probably, chemoprevention of HCC.

Key words: acyclic retinoid, branched-chain amino acids, hepatocellular carcinoma, phosphorylated retinoid X receptor-α, retinoic acid receptor-β

INTRODUCTION

THE POOR PROGNOSIS for patients with hepatocellular carcinoma (HCC) has created an urgent need to develop more effective strategies for prevention of this malignancy. Retinoids, which have tumor-suppressive and chemopreventive properties in various organs, are considered to be promising agents for improving outcomes in individuals with HCC.[1,2] A clinical trial demonstrated that the administration of acyclic retinoid (ACR), a synthetic retinoid that targets retinoid X receptor-α (RXR-α), significantly reduced the incidence of post-therapeutic recurrence of HCC.[3] ACR inhibits growth in human HCC cells by inducing apoptosis and arrest of the cell cycle in G_0–G_1.[4,5] The inhibition of growth in cancer cells by ACR is also associated with induction of cellular levels of retinoic acid receptor-β (RAR-β), an important retinoid receptor for regulation of apoptosis, and the inhibition of RXR-α phosphorylation.[5–8] The latter effect is more significant because the accumulation of phosphorylated (i.e. inactivated) RXR-α (p-RXR-α) interferes with the function of normal RXR-α in a dominant-negative manner, and therefore plays a critical role in the development of HCC.[2,9,10]

In addition, ACR acts synergistically with various agents (e.g. β-interferon, OSI-461, trastuzumab, valproic

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acid and vitamin K_2) that target other signaling pathways in suppressing growth and inducing apoptosis in human HCC cells.[5,11–14] These findings have clinical significance for the treatment and chemoprevention of HCC because the combined use of two or more agents can diminish drug toxicity while exerting synergistic effects. Branched-chain amino acids (BCAA; leucine, isoleucine and valine), which improve protein malnutrition in patients with liver cirrhosis, are candidate partners in ACR-based combination chemoprevention because a recent clinical trial showed that oral supplementation with these agents reduced the risk of HCC in obese patients with chronic viral liver disease.[15] Treatment with BCAA also prevents the development of liver tumorigenesis in a rodent model, while also inhibiting the growth of HCC cells.[16–18] The purpose of this study is to investigate whether the combination of ACR plus BCAA significantly inhibits the growth of human HCC xenografts and to examine the possible mechanisms of this action.

METHODS

Materials

AN ACYCLIC RETINOID (peretinoin) was supplied by Kowa Pharmaceutical (Tokyo, Japan). BCAA was obtained from Ajinomoto (Tokyo, Japan). The BCAA composition (2:1:1.2 = leucine : isoleucine : valine) was set at the clinical dose used for the treatment of decompensated liver cirrhosis in Japan.[15]

Experimental procedure

Thirty-two male BALB/c nude mice (5 weeks of age) were obtained from Charles River Japan (Tokyo, Japan). Xenograft tumors were made by the s.c. injection of Huh7 human HCC cells (Japanese Cancer Research Resources Bank, Tokyo, Japan) into the flanks of the mice at a concentration of 5×10^6 cells per 200 μL.[19] The mice were randomly divided into four groups (eight mice per group) 1 week after tumor cell injection. The mice in group 2 (ACR alone) were given the basal diet, CRF-1 (Oriental Yeast, Tokyo, Japan), containing 0.03% ACR with free access to feeding for 5 weeks. Group 3 (BCAA alone) was given the basal diet supplemented with 3.0% BCAA (w/w). The mice in group 4 (combination group) received a diet containing 0.03% ACR and 3.0% BCAA. Group 1 was given the basal diet and served as an untreated control. The tumor volume was calculated at the termination of the experiment using the formula: largest diameter × (smaller diameter)^2 × 0.5.

Protein extraction and western blot analysis

Total protein was extracted from the xenografts of Huh7 cells and equivalent amounts of protein (20 mg/lane) were examined by a western blot analysis.[19] The primary antibodies for RXR-α (ΔN-197 and D-20), extracellular signal-regulated kinase (ERK), phosphorylated ERK (p-ERK), Akt, phosphorylated Akt (p-Akt), insulin-like growth factor-1 receptor (IGF-1R), phosphorylated IGF-1R (p-IGF-1R) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) have been previously described.[5,10,19,20] The ΔN-197 antibody is regarded as a specific antibody for the phosphorylated form of RXR-α protein.[8,10] The intensities of the blots were quantified with NIH Image software ver. 1.62.

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

Total RNA was isolated from the xenografts of Huh7 cells using the RNAqueous-4PCR kit (Ambion Applied Biosystems, Austin, TX, USA). The cDNA was amplified from 0.2 μg of total RNA using SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). The primers used for the amplification of RAR-β, $p21^{CIP1}$ and GAPDH-specific genes have been previously described.[6,19] A quantitative real-time RT–PCR analysis was performed in a LightCycler (Roche Diagnostics, Mannheim, Germany) with SYBR Premix Ex Taq (TaKaRa Bio, Shiga, Japan).[16] The gene expression levels were normalized to the GAPDH expression levels using a standard curve.

Statistical analysis

The data are expressed as the mean ± standard deviation. Statistical significance of the difference in mean values was assessed by one-way ANOVA, followed by Scheffé's *t*-test.

RESULTS

Combined treatment with ACR plus BCAA significantly inhibits growth of HCC xenografts

AS SHOWN IN Figure 1, neither treatment with 0.03% ACR alone nor 3.0% BCAA alone inhibited the growth of Huh7 xenografts. These findings suggest that such doses of ACR and BCAA are insufficient to suppress the tumor growth of HCC in the present study, although similar concentrations of these agents have had a significant effect on preventing the development

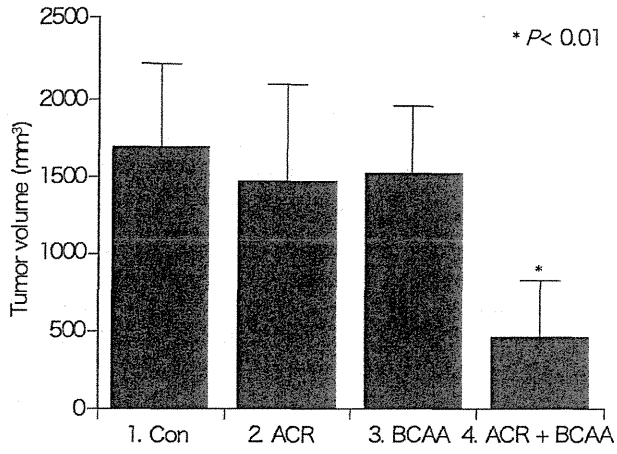

Figure 1 Effects of the combination of acyclic retinoid (ACR) plus branched-chain amino acid (BCAA) on the growth of Huh7 xenografts. BALB/c nude mice were injected s.c. with 5×10^6 Huh7 cells. One week after the injection, the mice were divided into four groups and treated as follows for 5 weeks: group 1, untreated control group (Con); group 2, 0.03% ACR-treated group; group 3, 3.0% BCAA-treated group; group 4, 0.03% ACR and 3.0% BCAA-treated group. The tumor volumes in each group at the termination of experiment are represented. Bars, standard deviation. *$P < 0.01$, significant differences obtained by comparisons to groups 1, 2, and 3.

of HCC in clinical trials.[3,15] On the other hand, the simultaneous treatment of the mice with these concentrations of ACR plus BCAA produced a significant decrease in the growth of HCC xenografts; the tumor volume was inhibited by 73% in the combination treatment group in comparison to the control group ($P < 0.01$).

BCAA inhibits the phosphorylation of Akt and IGF-1R, and enhances the suppression of the RXR-α and ERK phosphorylation by ACR in HCC xenografts

Retinoid X receptor-α phosphorylation by Ras/mitogen-activated protein kinase is closely associated with the development of HCC, and thus might be a critical target for chemoprevention of HCC.[2,9] BCAA inhibits the activation of IGF-1R and Akt and this is associated with the cancer chemopreventive effects of this agent.[16,21] Therefore, the combined effects of ACR plus BCAA on the phosphorylation of RXR-α, ERK, Akt and IGF-1R proteins were investigated in Huh7 xenografts. The expression levels of p-RXR-α and p-ERK proteins, which decreased in the ACR alone-treated group in comparison to the control group (Fig. 2a, column 2), decreased to a greater extent when the mice were treated with the combination of ACR plus BCAA (Fig. 2a, column 4). The expression levels of p-Akt and p-IGF-1R proteins, which were decreased in the BCAA alone-treated group (Fig. 2a, column 3), were also further reduced by combined treatment with ACR plus BCAA (Fig. 2a, column 4).

Combined treatment of ACR plus BCAA induces the RAR-b and $p21^{CIP1}$ mRNA in HCC xenografts

The combined effect of ACR plus BCAA on the induction of the RAR-β and $p21^{CIP1}$ mRNA was next examined because, in addition to the inhibition of RXR-α phosphorylation, ACR is known to inhibit the growth of HCC cells by enhancing the expression of these molecules.[4,5,7] Semiquantitative RT–PCR analyses showed that treatment with both ACR alone and BCAA alone tended to increase the levels of RAR-β mRNA, but the differences were not significant (Fig. 2b, columns 2 and 3). On the other hand, when ACR was combined with BCAA, the expression levels of this mRNA were significantly enhanced in comparison to the control group (Fig. 2b, column 4). In addition, treatment with ACR alone and the combination of ACR plus BCAA significantly increased the expression of $p21^{CIP1}$ mRNA (Fig. 2c, columns 2 and 4), a negative modulator of cell cycle progression,[22] in comparison to the control group.

DISCUSSION

COMBINATION CHEMOPREVENTION IS often advantageous because it provides the potential for additive or, in some instances, synergistic effects between specific agents. The present study clearly indicated that the combination of ACR plus BCAA, both of which exert chemopreventive properties on HCC development,[3,15] causes potent inhibition of growth in human HCC xenografts. The hypotheses that explain this beneficial effect are summarized in Figure 3.

Initially, it should be emphasized that the phosphorylation of RXR-α and ERK proteins was strongly inhibited by the combination of ACR plus BCAA. This study and prior ones[5,8,14,23] show that ACR alone inhibits the phosphorylation of these proteins, thus indicating that BCAA could enhance the effect of ACR in HCC xenografts. These findings seem to be significant because restoration of the function of RXR-α as a master regulator of nuclear receptors by targeting its phosphorylation might be an effective strategy for the prevention and treatment of HCC.[2] BCAA may support the effect of

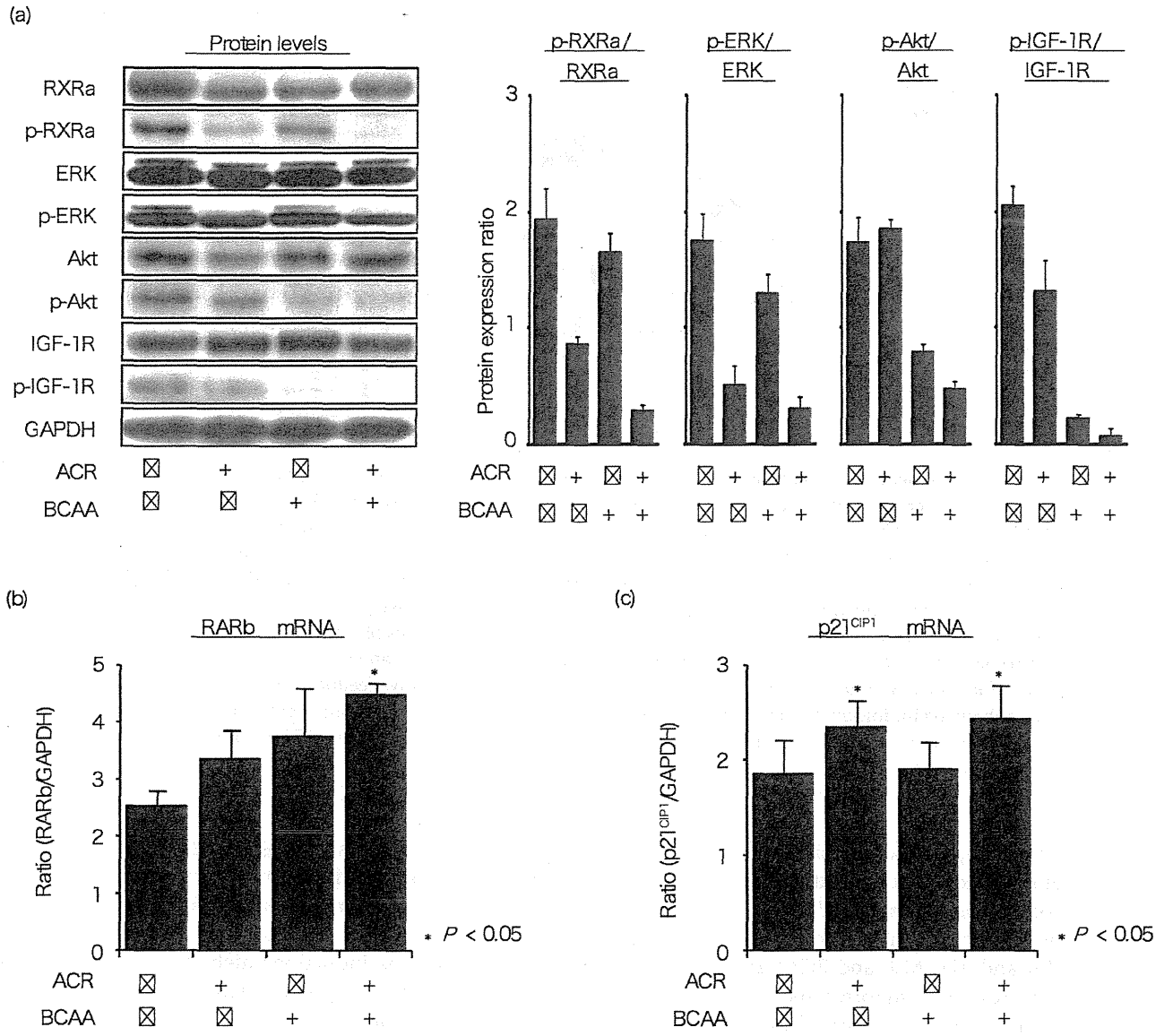

Figure 2 Effects of the combination of acyclic retinoid (ACR) plus branched-chain amino acid (BCAA) on phosphorylation of retinoid X receptor-α (RXR-α), extracellular signal-regulated kinase (ERK), Akt and insulin-like growth factor-1 receptor (IGF-1R) proteins and expression levels of retinoic acid receptor-β (RAR-β) and $p21^{CIP1}$ mRNA in Huh7 xenografts. The xenografts were excised from each animal at the termination of the experiment and tumor extracts were examined by a western blot analysis using the respective antibodies (a) or were examined by a quantitative real-time reverse transcription polymerase chain reaction analysis using RAR-β (b) and $p21^{CIP1}$ (c) specific primers. (a) Western blot analysis for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was performed using a single membrane and equal protein loading was verified by the detection of this protein. Repeat western blots yielded similar results. Lanes, protein samples from each group (left). The intensities of blots were quantitated by densitometry (right). (b,c) The expression levels of *RAR-b* (b) and *$p21^{CIP1}$* (c) genes were normalized to GAPDH expression. Bars, standard deviations of triplicate assays. *$P < 0.05$, significant differences obtained by comparison to the control group (group 1). p-, phosphorylated.

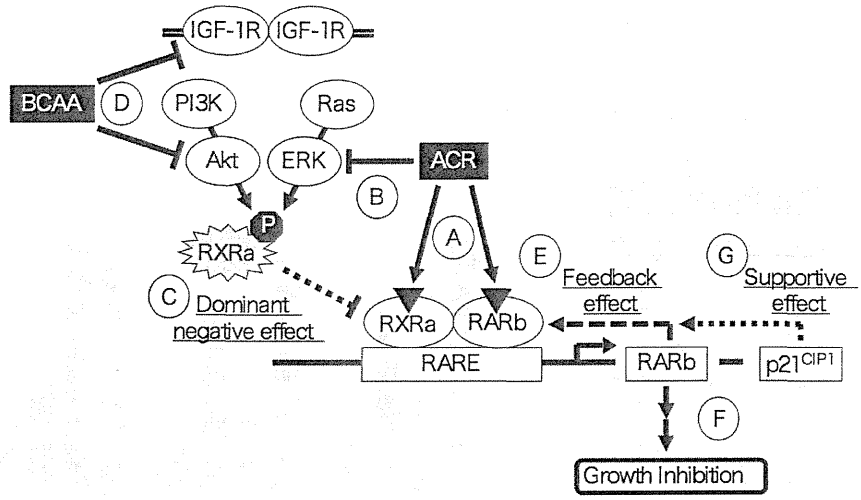

Figure 3 Hypothetical schematic representation of the effect of the combination of acyclic retinoid (ACR) plus branched-chain amino acid (BCAA) on growth inhibition in hepatocellular carcinoma (HCC) xenografts. ACR can bind to both retinoic acid receptor (RAR) and retinoid X receptor (RXR) as a ligand (a), and activate the retinoic acid responsive element (RARE) promoter activity, thus increasing the levels of both RAR-β and $p21^{CIP1}$ because the promoter region of these molecules contains RARE. In parallel, ACR inactivates the Ras/mitogen-activated protein kinase signaling pathways (b). This signaling pathway phosphorylates RXR-α, and thus impairs the function of this receptor in a dominant-negative manner (c). On the other hand, BCAA inhibits the activation of IGF-1R and its downstream Akt, which is also involved in RXR-α phosphorylation (d). Cooperative inhibition of RXR-α phosphorylation by ACR plus BCAA might restore the function of this receptor and subsequently increase RAR-β expression. This induction of RAR-β and its activation by the ligand ACR might produce a positive feedback effect on the expression of RAR-β itself (e), thus enhancing inhibition of growth in HCC cells (f). Induction of $p21^{CIP1}$ might support this positive feedback effect (g). For additional details see the "Discussion" section. ERK, extracellular signal-regulated kinase; IGF-1R, insulin-like growth factor-1 receptor.

ACR, at least in part, by inhibiting the activation of IGF-1R and its downstream Akt, because some types of receptor tyrosine kinases (RTK), including IGF-1R, might phosphorylate RXR-α through the phosphorylation of ERK and Akt. ACR and BCAA reduce the development of HCC and suppress the growth of cancer cells by inhibiting the activation of specific RTK, including IGF-1R and epidermal growth factor receptor (EGFR).[6,16,24] BCAA also suppresses insulin-induced hepatic tumor cell proliferation by inhibiting ERK and Akt phosphorylation.[18] The previous reports showing that there is a cross-talk between EGFR and IGF-1R, and that the simultaneous targeting of these RTK induces a synergistic inhibition of growth in HCC cells, might give this hypothesis credibility.[25,26]

The reduction in the dominant negative effect of RXR-α phosphorylation by combining ACR plus BCAA might activate the transcriptional activity of retinoic acid responsive element (RARE).[5,10] This is associated with the increased expression of RAR-β and $p21^{CIP1}$ mRNA because the promoter region of these genes contains RARE.[27,28] RAR-β, which is also a receptor for ACR, can exert tumor-suppressive effects in cancer cells.[29] Therefore, the induction of RAR-β by the treatment with ACR plus BCAA might have played a critical role in inhibiting the growth of HCC xenografts in the present study. In addition, this induction might be, at least in part, associated with $p21^{CIP1}$ upregulation by ACR plus BCAA because introduction of the *$p21^{CIP1}$* gene into cells induces RAR-β expression and sensitizes cancer cells to retinoid treatment.[30] This hypothesis may be supported by recent reports that a substantial induction of RAR-β and $p21^{CIP1}$ produces positive feedback effects on the expression of RAR-β.[5,12]

Acyclic retinoid has an agonistic activity for both RAR and RXR.[2] Therefore, the reduction of the dominant-negative effect of RXR-α phosphorylation and the induction of the RAR-β expression by the combination of ACR plus BCAA might exert a significant inhibition of growth in the HCC xenografts. Because this study shows the possibility that the combination treatment consisting of ACR plus BCAA is an effective regimen for the treatment of HCC, we presume that this combination might also be useful for the prevention of HCC. In order to confirm

this prediction, future studies are required to determine whether this combination treatment prevents the development of HCC using chemically-induced liver carcinogenesis in a rodent model with, for example, diethylnitrosamine.

In conclusion, this study, as well as prior ones,[5,11–14] indicates that the combination chemoprevention using ACR as a key agent might be an effective strategy for the prevention and treatment of HCC. Among such regimens, particularly combining ACR with BCAA might hold promise as a clinical modality for the chemoprevention of HCC because clinical trials have shown that both of these agents can significantly prevent the development of HCC without causing any adverse effects.[3,15]

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Synergistic growth inhibition of human hepatocellular carcinoma cells by acyclic retinoid and GW4064, a farnesoid X receptor ligand

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ABSTRACT

Abnormalities in the expression and function of retinoid X receptor (RXR), a master regulator of the nuclear receptor superfamily, are associated with the development of hepatocellular carcinoma (HCC). Dysfunction of farnesoid X receptor (FXR), one of the nuclear receptors that forms a heterodimer with RXR, also plays a role in liver carcinogenesis. In the present study, we examined the effects of acyclic retinoid (ACR), a synthetic retinoid targeting RXRα, plus GW4064, a ligand for FXR, on the growth of human HCC cells. We found that ACR and GW4064 preferentially inhibited the growth of HLE, HLF, and Huh7 human HCC cells in comparison with Hc normal hepatocytes. The combination of 1 μM ACR plus 1 μM GW4064 synergistically inhibited the growth of HLE cells by inducing apoptosis. The combined treatment with these agents acted cooperatively to induce cell cycle arrest in the G_0/G_1 phase and inhibit the phosphorylation of RXRα, which is regarded as a critical factor for liver carcinogenesis, through inhibition of ERK and Stat3 phosphorylation. This combination also increased the expression levels of $p21^{CIP1}$ and SHP mRNA, while decreasing the levels of c-*myc* and cyclin D1 mRNA in HLE cells. In addition, a reporter assay indicated that the FXRE promoter activity was significantly increased by treatment with ACR plus GW4064. Our results suggest that ACR and GW4064 cooperatively inhibit RXRα phosphorylation, modulate the expression of FXR-regulated genes, thus resulting in the induction of apoptosis and the inhibition of growth in HCC cells. This combination might therefore be effective for the chemoprevention and chemotherapy of HCC.

1. Introduction

Nuclear receptors are ligand-dependent transcription factors that are involved in various physiological processes. Retinoid X receptors (RXRs) are regarded as master regulators of nuclear receptors because they play an essential role in controlling normal cell proliferation and metabolism by forming a heterodimer with other nuclear receptors [1,2]. Therefore, abnormalities in the expression and function of RXRs are closely associated with the development of various disorders, including cancer, whereas using a retinoid might be an effective strategy for the prevention and treatment of human malignancies [3]. A malfunction of RXRα, one of the subtypes of RXR, due to phosphorylation by the Ras/MAPK signaling pathway is profoundly associated with liver carcinogenesis [4–8]. On the other hand, administration of acyclic retinoid (ACR), a synthetic retinoid which targets RXRα, reduced the incidence of post-therapeutic recurrence of hepatocellular carcinoma (HCC) and improved the survival rate of patients with this malignancy [9,10]. ACR also inhibits the growth of HCC-derived cells by inducing apoptosis and cell cycle arrest in the G_0/G_1 phase [11,12]. These findings suggest that nuclear receptors, especially RXRα, are critical targets for the prevention and treatment of HCC.

Farnesoid X receptor (FXR), which has been characterized as a bile acid receptor, is also a member of the nuclear receptor superfamily of ligand-dependent transcription factors that form heterodimers with RXR [13]. FXR has been shown to be essential in controlling bile acid, lipid, and glucose homeostasis [13]. It also plays a critical role in normal liver regeneration and promotes liver repair after injury by mediating its related signaling pathways [14].

Abbreviations: ACR, acyclic retinoid; CI, combination index; DAPI, 4',6-diamidino-2-phenylindole; ERK, extracellular signal-regulated kinase; FXR, farnesoid X receptor; FXRE, farnesoid X receptor response element; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HCC, hepatocellular carcinoma; IFN, interferon; MAPK, mitogen-activated protein kinase; PARP, poly (ADP-ribose) polymerase; RAR, retinoic acid receptor; RARE, retinoic acid response element; RTK, receptor tyrosine kinase; RT-PCR, reverse transcription PCR; RXR, retinoid X receptor; SHP, small heterodimer partner; Stat3, signal transducer and activator of transcription 3; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling.

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