

# Pitavastatin inhibits azoxymethane-induced colonic preneoplastic lesions in C57BL/KsJ-*db/db* obese mice

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Obesity and related metabolic abnormalities are risk factors for colorectal cancer. A state of chronic inflammation and adipocytokine imbalance may play a role in colorectal carcinogenesis. Statins, which are commonly used for the treatment of hyperlipidemia, are known to possess anti-inflammatory effects. Statins also exert chemopreventive properties against various cancers. The present study examined the effects of pitavastatin, a recently developed lipophilic statin, on the development of azoxymethane (AOM)-initiated colonic premalignant lesions in C57BL/KsJ-*db/db* (*db/db*) obese mice. Male *db/db* mice were administered weekly subcutaneous injections of AOM (15 mg/kg body weight) for 4 weeks and then were subsequently fed a diet containing 1 ppm or 10 ppm pitavastatin for 8 weeks. Feeding with either dose of pitavastatin significantly reduced the number of colonic premalignant lesions,  $\beta$ -catenin accumulated crypts, by inhibiting proliferation and the surrounding inflammation. Pitavastatin increased the serum levels of adiponectin while conversely decreasing the serum levels of total cholesterol, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin (IL)-6, IL-18, and leptin. Pitavastatin also caused a significant increase in the expression of phosphorylated form of the AMP-activated kinase (AMPK) protein on the colonic mucosa of AOM-treated mice. In addition, the expression levels of TNF- $\alpha$ , IL-6, IL-18, and COX-2 mRNAs on the colonic mucosa of AOM-treated mice were decreased by treatment with this agent. These findings suggest that pitavastatin attenuates chronic inflammation and improves the imbalance of adipocytokines, both of which are caused by the presence of excess adipose tissues, thereby preventing the development of colonic premalignancies in an obesity-related colon cancer model. Therefore, some types of statins, including pitavastatin, may be a useful chemoprevention modality for colon cancer in obese individuals. (*Cancer Sci* 2010; 101: 1701-1707)

Colorectal cancer (CRC) is a serious healthcare problem worldwide due to its substantial morbidity and mortality rates in patients. Recent evidence has indicated that obesity and its related metabolic abnormalities are associated with an increased risk for CRC.<sup>(1-3)</sup> Several hypotheses have emerged to explain the influence of obesity on the development of CRC, including insulin resistance, alterations in the insulin-like growth factor (IGF)/IGF-I receptor axis and adipocytokine imbalance, such as increased leptin levels and decreased adiponectin levels.<sup>(1-3)</sup> A state of chronic inflammation, which is induced by excessive production of storage lipids and high circulating glucose levels, also plays a critical role in obesity-related colorectal carcinogenesis.<sup>(4)</sup> Adipose tissue constitutively expresses the pro-inflammatory cytokine tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ).<sup>(5,6)</sup> Because TNF- $\alpha$ , a central mediator in chronic inflammatory diseases, stimulates tumor promotion and progression of carcinogenesis, the inhibition of this key pro-inflammatory molecule may therefore be a novel treatment

and prevention strategy for several malignancies, including CRC.<sup>(7,8)</sup>

Statins, 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) reductase inhibitors, are widely used for the treatment of hyperlipidemia and have been shown to exert beneficial effects on both cardiovascular and cerebrovascular diseases.<sup>(9,10)</sup> Statins prevent the conversion of HMG-CoA to mevalonate and thus decrease serum lipid levels, especially those of low-density lipoprotein cholesterol and triglyceride. In addition to their lipid-lowering effect, statins have demonstrated anticancer properties.<sup>(11,12)</sup> Statins suppress inflammation, induce apoptosis, and modulate angiogenesis, thus inhibiting the growth of a wide variety of cancer cells.<sup>(11,12)</sup> Statins induce apoptosis in human colon cancer cells and attenuate inflammation-related colon carcinogenesis in mice.<sup>(13,14)</sup> Epidemiologic studies have also demonstrated the chemopreventive properties of statins for various types of cancer, including CRC.<sup>(11,12,15)</sup> These reports suggest that statin administration might therefore be an effective strategy for preventing the development of CRC. However, no detailed studies on whether statins can prevent the development of obesity-related CRC have so far been conducted.

In order to develop an effective method to prevent obesity-related colorectal carcinogenesis, we established a useful preclinical animal model using the colonic carcinogen azoxymethane (AOM) and C57BL/KsJ-*db/db* (*db/db*) mice, which are obese and exhibit hyperlipidemia.<sup>(16-19)</sup> In the present study, we investigated the effects of pitavastatin, a recently developed lipophilic statin,<sup>(20)</sup> on the development of  $\beta$ -catenin accumulated crypts (BCAC) and aberrant crypt foci (ACF), both of which are putative precursor lesions for colonic adenocarcinoma<sup>(21,22)</sup> and which are utilized as biomarkers to evaluate a number of agents for their potential chemopreventive properties,<sup>(23,24)</sup> in *db/db* mice treated with AOM.

## Materials and Methods

**Animals, chemicals, and diets.** Four-week-old male homozygous *db/db* mice were obtained from Japan SLC (Shizuoka, Japan). All mice were maintained at the Gifu University Life Science Research Center in accordance with the Institutional Animal Care Guidelines. Azoxymethane (AOM) was purchased from Sigma Chemical (St. Louis, MO, USA). Pitavastatin was obtained from Kowa Pharmaceutical (Tokyo, Japan).

**Experimental procedure.** The animal experiment, as described previously,<sup>(18,19)</sup> was approved by the Committee of the Institutional Animal Experiments of Gifu University. A total of 34 male *db/db* mice were divided into five groups. At 5 weeks of age, the mice in Groups 1 (five mice) and 2 (five mice) were given four weekly subcutaneous injections of saline. The mice in Groups 3 (eight mice), 4 (eight mice), and 5 (eight mice) were

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subcutaneously injected with AOM (15 mg/kg body weight) once a week for 4 weeks. Groups 1 and 3 were fed the basal diet CRF-1 (Oriental Yeast, Tokyo, Japan), throughout the experiment (for 12 weeks). Group 2 was fed the diet containing 10 ppm pitavastatin throughout the experiment. Groups 4 and 5 were given the diets containing 1 and 10 ppm pitavastatin, respectively, for 8 weeks, starting 1 week after the last injection of AOM. At the termination of the study (17 weeks of age), all mice were sacrificed by CO<sub>2</sub> asphyxiation for analyzing histopathology, the number of colonic BCAC and ACF, and clinical chemistry.

**Counting the number of BCAC and ACF.** The frequencies of BCAC and ACF were determined according to the standard procedures described previously.<sup>(17-19)</sup> After the resected colons on filter papers were fixed in 10% buffered formalin for 24 h, the mucosal surfaces were stained with methylene blue (0.5% in distilled water) and the number of ACF was counted under a light microscope. After counting the ACF, the distal parts (1 cm from anus) of the colon were cut to count the number of BCAC. To identify BCAC intramucosal lesions, the distal part of the colon (mean area, 0.7 cm<sup>2</sup>/colon) was embedded in paraffin and a total of 20 serial sections (4- $\mu$ m thick each) per mouse were created by an *en face* preparation.<sup>(17-19)</sup> For each case, two serial sections were used to analyze BCAC.

**Histopathology and immunohistochemical analyses for  $\beta$ -catenin and PCNA.** Three serial sections were cut from paraffin-embedded tissue blocks. Two sections were subjected to hematoxylin-eosin (H&E) staining for histopathology and  $\beta$ -catenin immunohistochemistry to count the number of BCAC. The other section was used for immunohistochemical staining of proliferating cell nuclear antigen (PCNA), a G<sub>1</sub>-to-S phase marker, to estimate the proliferative activity of the BCAC. Immunohistochemical staining of  $\beta$ -catenin and PCNA were performed using the labeled streptavidin-biotin method (LSAB kit; Dako, Glostrup, Denmark) as previously described.<sup>(17-19)</sup> The primary antibodies included an anti- $\beta$ -catenin antibody (1:1000 final dilution) from BD Transduction Laboratories (Cat. No. 610154; San Jose, CA, USA) and an anti-PCNA antibody (1:100 final dilution) from Santa Cruz Biotechnology (sc-7907; Santa Cruz, CA, USA). The negative control sections were immunostained without any primary antibodies. On the PCNA-immunostained sections, the cells with intensively reacted nuclei were considered to be positive for PCNA, and the indices (%) were calculated in randomly selected 10 BCAC from Group 3, five BCAC from Group 4, and five BCAC from Group 5. The PCNA-labeling indices for both ACF and surrounding crypts were also determined by representative lesions ( $n = 8$ ), respectively.

**RNA extraction and quantitative real-time RT-PCR.** The expression levels of the *TNF- $\alpha$* , interleukin (*IL*)-6, *IL*-18, and cyclooxygenase (*COX*)-2 genes were determined in the colonic mucosa of mice from Groups 3 through 5. A quantitative real-time RT-PCR analysis was performed as described previously.<sup>(25)</sup> Total RNA was isolated from the scraped colonic

mucosa of the experimental mice using the RNAqueous-4PCR kit (Ambion Applied Biosystems, Austin, TX, USA). The cDNA was synthesized from 0.2  $\mu$ g of total RNA using the SuperScript III First-Strand Synthesis System (Invitrogen, San Diego, CA, USA). The specific primers used for the amplification of *TNF- $\alpha$* , *IL*-6, *IL*-18, *COX*-2, and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) genes are as previously described.<sup>(8)</sup> Real-time RT-PCR was performed in a LightCycler (Roche Diagnostics, Indianapolis, IN, USA) with the SYBR Premix Ex Taq (Takara Bio, Shiga, Japan). The expression levels of the *TNF- $\alpha$* , *IL*-6, *IL*-18, and *COX*-2 genes were normalized to the *GAPDH* gene expression levels.

**Protein extraction and western blot analysis.** Total proteins were extracted from the scraped mucosa from the remaining colon of the AOM-treated mice (Groups 3 through 5) and equivalent amounts of proteins (20  $\mu$ g/lane) were examined by western blot analysis.<sup>(18,19)</sup> The primary antibodies for AMP-activated kinase (AMPK) and the phosphorylated form of AMPK (p-AMPK) were obtained from Cell Signaling Technology (Danvers, MA, USA). An antibody against GAPDH (Chemicon International, Temecula, CA, USA) served as a loading control.

**Clinical chemistry.** After 6 h of fasting, blood samples were collected at the time of sacrifice to measure the serum concentrations of total cholesterol, triglyceride, adiponectin, leptin, *TNF- $\alpha$* , *IL*-6, and *IL*-18. The serum total cholesterol and triglyceride levels were assayed as described previously.<sup>(17-19)</sup> The serum adiponectin (Otsuka, Tokyo, Japan), leptin (R&D Systems, Minneapolis, MN, USA), *TNF- $\alpha$*  (Shibayagi, Gunma, Japan), *IL*-6 (IBL, Gunma, Japan), and *IL*-18 (MBL, Aichi, Japan) levels were determined by an enzyme immunoassay (EIA) according to the manufacturer's protocol.

**Statistical analyses.** The results are presented as the mean  $\pm$  SD and were analyzed using the GraphPad InStat software program, version 3.05 (GraphPad Software, San Diego, CA, USA) for Macintosh. The differences between groups were analyzed by one-way ANOVA or, as required, by two-way ANOVA. When ANOVA showed a statistically significant effect ( $P < 0.05$ ), comparisons of each experimental group with the control group were performed using the Tukey-Kramer multiple comparisons test. The differences were considered statistically significant when the two-tailed  $P$ -value was  $< 0.05$ .

## Results

**General observations.** As shown in Table 1, the average body weights of the AOM-injected groups (Groups 3 through 5) at the termination of the experiment were significantly lower than that of the saline-injected group (Group 1,  $P < 0.05$ , respectively). This might be caused by the toxicity of AOM as observed in previous experiments.<sup>(18,19)</sup> No significant differences were observed in the mean weights of the liver and kidney among the groups. A histopathological examination revealed the absence of

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

Group no.	Treatment	No. of mice	Final body wt (g)	Relative organ weight (g/100 g body wt)	
				Liver	Kidney
1	Saline	5	51.2 $\pm$ 8.4 <sup>†</sup>	6.23 $\pm$ 1.55	0.85 $\pm$ 0.25
2	Saline + 10 ppm pitavastatin	5	49.2 $\pm$ 7.7	6.05 $\pm$ 0.88	0.98 $\pm$ 0.23
3	AOM alone	8	40.9 $\pm$ 6.5*	4.69 $\pm$ 1.10	0.95 $\pm$ 0.11
4	AOM + 1 ppm pitavastatin	8	41.1 $\pm$ 8.5*	5.28 $\pm$ 0.79	1.05 $\pm$ 0.17
5	AOM + 10 ppm pitavastatin	8	39.7 $\pm$ 6.9*	5.14 $\pm$ 1.02	1.10 $\pm$ 0.25

\*Significantly different from Group 1 ( $P < 0.05$ ). <sup>†</sup>Mean  $\pm$  SD. AOM, azoxymethane.

toxicity of pitavastatin in these tissues of the mice that received pitavastatin (Groups 2, 4, and 5). As listed in Table 2, the average colon lengths in Groups 3 through 5 were shorter than that of Group 1 ( $P < 0.05$ , respectively). However, treatment with pitavastatin did not affect the colon length.

**Effects of pitavastatin on AOM-induced BCAC and ACF formations in the *db/db* mice.** Table 2 summarizes the total numbers of BCAC and ACF in each group. Aberrant crypt foci (ACF) (Fig. 1a,b) and BCAC (Fig. 1c–f) developed in the colons of all mice that received AOM (Groups 3 through 5), but not in those treated without AOM (Groups 1 and 2). The sizes of BCAC and ACF that developed in Group 3 (Fig. 1a,c) tended to be larger compared to those of Groups 4 and 5 (Fig. 1b,d). Interestingly, the inflammatory cell infiltration surrounding BCAC that developed in Group 3 (Fig. 1c) also tended to be more severe compared to that in Groups 4 and 5 (Fig. 1d). The total number of ACF in Groups 4 and 5 was smaller than that of Group 3, but the differences were not statistically significant. As for the total number of BCAC, the values of Groups 4 ( $P < 0.05$ ) and 5 ( $P < 0.01$ ) were significantly lower than that of Group 3: the inhibition rates were 60% in Group 4 and 75% in Group 5, respectively (Table 2).

**Effects of pitavastatin on the proliferation activity in BCAC of the AOM-injected *db/db* mice.** The PCNA-labeling index of BCAC developed in the AOM-treated *db/db* mice was deter-

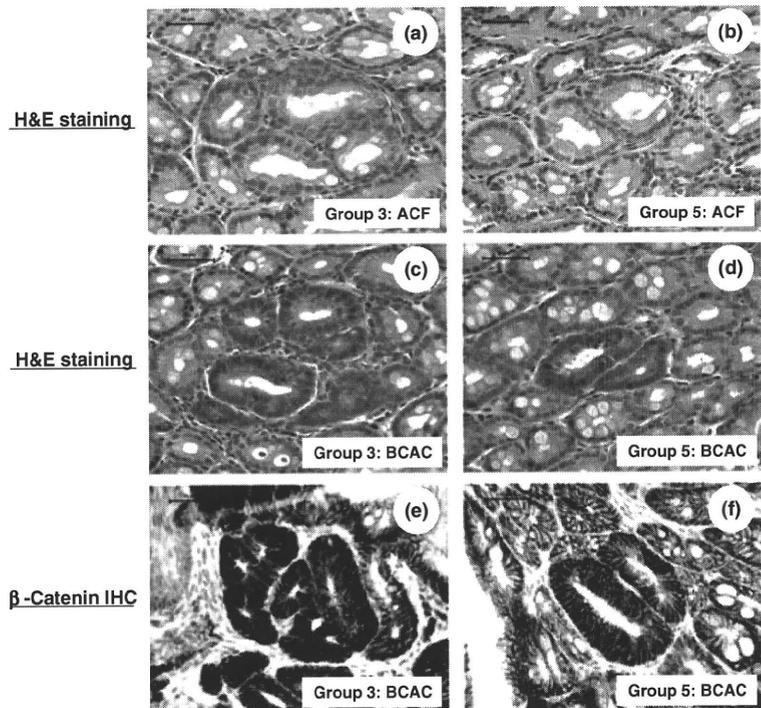
mined by the PCNA-immunohistochemical sections (Fig. 2a–c). As illustrated in Figure 2(d), the mean PCNA-labeling index in the 1 ppm ( $78.7 \pm 6.6\%$ ) and 10 ppm ( $71.1 \pm 13.4\%$ ) pitavastatin-treated mice was significantly smaller than that in the mice which received AOM alone ( $95.3 \pm 3.0\%$ ;  $P < 0.01$  for each comparison). Conversely, neither the PCNA-labeling indices of ACF ( $40.6 \pm 5.9\%$ ) nor the surrounding crypts ( $23.1 \pm 9.5\%$ ) were affected by the treatment with 1 ppm ( $38.6 \pm 7.0\%$  or  $24.6 \pm 5.2\%$ ) or 10 ppm pitavastatin ( $34.9 \pm 8.5\%$  or  $22.4 \pm 8.8\%$ ), respectively. These findings indicate that pitavastatin significantly suppresses BCAC in comparison to ACF, at least in part, by reducing cell proliferation selectively in the lesion.

**Effects of pitavastatin on serum levels of total cholesterol, triglyceride, adiponectin, and leptin in the *db/db* mice.** The serum concentrations of the total cholesterol, triglyceride, adiponectin, and leptin levels are listed in Table 3. Although there were no significant differences in the serum triglyceride levels, the serum levels of total cholesterol in the pitavastatin-treated groups (Groups 2, 4, and 5) were significantly lower than those in Groups 1 and 3, regardless of AOM-injection ( $P < 0.05$  and  $P < 0.01$ , respectively). The mice treated with both doses of pitavastatin showed a significant increase in the serum levels of adiponectin when compared to the mice treated without pitavastatin, regardless of AOM-injection ( $P < 0.05$  and  $P < 0.05$ ,

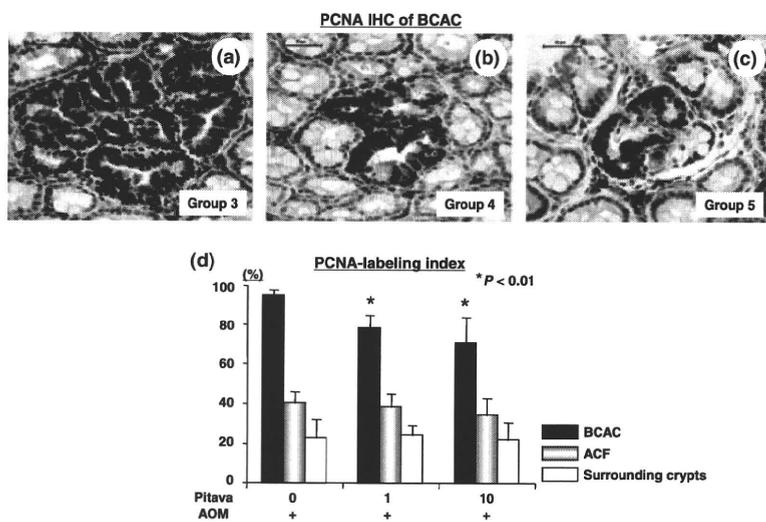
**Table 2. Effects of pitavastatin on AOM-induced ACF and BCAC formation in the experimental mice**

Group no.	Treatment	No. of mice	Length of colon (cm)	Total no. of ACFs/colon	Total no. of BCACs/cm <sup>2</sup>
1	Saline	5	12.2 ± 0.8 <sup>†</sup>	0	0
2	Saline + 10 ppm pitavastatin	5	11.7 ± 1.0	0	0
3	AOM alone	8	11.1 ± 0.7*	80.4 ± 13.4	13.6 ± 9.3
4	AOM + 1 ppm pitavastatin	8	11.7 ± 0.7*	73.5 ± 11.4	5.4 ± 1.8**
5	AOM + 10 ppm pitavastatin	8	11.0 ± 1.2*	72.8 ± 15.2	3.4 ± 2.6***

\*Significantly different from Group 1 ( $P < 0.05$ ). \*\*Significantly different from Group 3 ( $P < 0.05$ ). \*\*\*Significantly different from Group 3 ( $P < 0.01$ ). <sup>†</sup>Mean ± SD. ACF, aberrant crypt foci; AOM, azoxymethane; BCAC,  $\beta$ -catenin accumulated crypts.



**Fig. 1.** The representative histopathology and  $\beta$ -catenin-immunohistochemistry of aberrant crypt foci (ACF) and  $\beta$ -catenin accumulated crypts (BCAC). (a,b) Aberrant crypt foci (ACF) on the H&E-stained sections from Groups 3 and 5. (c,d)  $\beta$ -Catenin accumulated crypts (BCAC) on the H&E-stained sections from Groups 3 and 5. (e,f)  $\beta$ -Catenin-immunohistochemistry of BCAC from Groups 3 and 5. Note: The sizes of ACF and BCAC developed in Group 5 are smaller than those of Group 3. The localization of the accumulated  $\beta$ -catenin protein is apparent in the cytoplasm and nucleus of atypical cryptal cells in BCAC. IHC, immunohistochemistry. Scale bar, 50  $\mu$ m.



**Fig. 2.** The effect of pitavastatin on cell proliferation in  $\beta$ -catenin accumulated crypts (BCAC) and aberrant crypt foci (ACF) induced by azoxymethane (AOM) in male *db/db* mice. (a-c) Representative proliferating cell nuclear antigen (PCNA)-immunohistochemistry of the BCAC developed in the colon of Groups 3, 4, and 5. (d) The mean PCNA-labeling indices of the BCAC, ACF, and surrounding crypts in the colon of Groups 3 through 5. IHC, immunohistochemistry. Scale bar, 50  $\mu$ m. \* $P < 0.01$  vs Group 3.

**Table 3.** Serum levels of total cholesterol, triglyceride, adiponectin, and leptin in the experimental mice

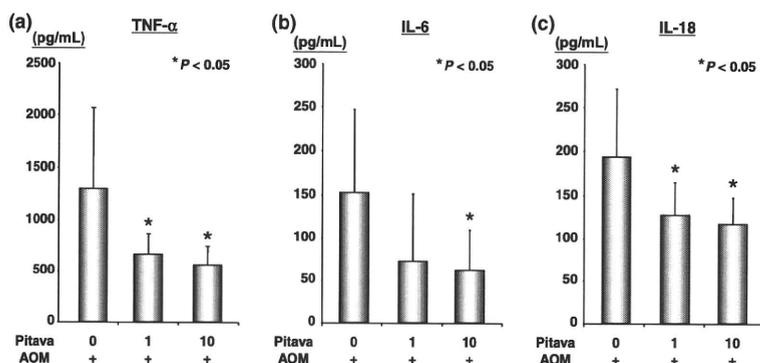
Group no.	Treatment	No. of mice	Total cholesterol (mg/dL)	Triglyceride (mg/dL)	Adiponectin ( $\mu$ g/mL)	Leptin (ng/dL)
1	Saline	5	283 $\pm$ 44 $\dagger$	168 $\pm$ 42	11.2 $\pm$ 1.7	87.0 $\pm$ 14.4
2	Saline + 10 ppm pitavastatin	5	193 $\pm$ 58*	174 $\pm$ 29	15.7 $\pm$ 1.7*	72.6 $\pm$ 15.1
3	AOM alone	8	170 $\pm$ 13**	132 $\pm$ 24	13.1 $\pm$ 1.3	93.6 $\pm$ 4.2
4	AOM + 1 ppm pitavastatin	8	143 $\pm$ 17***	138 $\pm$ 24	18.0 $\pm$ 3.2****	80.6 $\pm$ 6.5***
5	AOM + 10 ppm pitavastatin	8	133 $\pm$ 22***	133 $\pm$ 25	17.7 $\pm$ 3.0****	70.7 $\pm$ 19.9***

\*Significantly different from Group 1 ( $P < 0.05$ ). \*\*Significantly different from Group 1 ( $P < 0.01$ ). \*\*\*Significantly different from Group 3 ( $P < 0.01$ ). \*\*\*\*Significantly different from Group 3 ( $P < 0.05$ ).  $\dagger$ Mean  $\pm$  SD. AOM, azoxymethane.

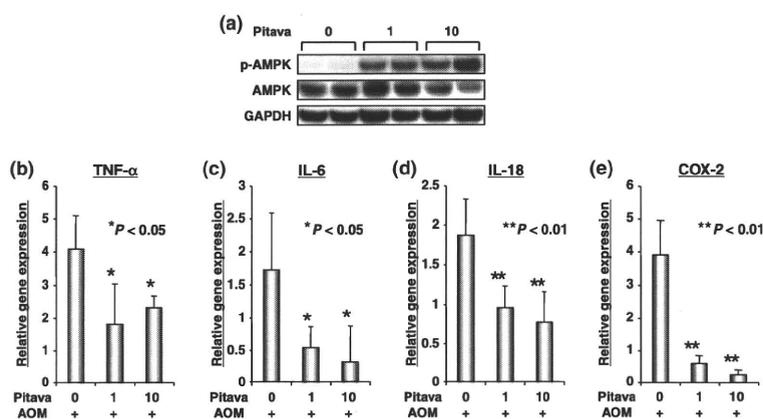
respectively). In addition, the serum leptin levels of 1 ppm and 10 ppm pitavastatin-treated mice were significantly lower than those in untreated control mice ( $P < 0.01$  for each comparison).

**Effects of pitavastatin on the serum level of TNF- $\alpha$ , IL-6, and IL-18 in the *db/db* mice.** The effects of pitavastatin on the serum levels of TNF- $\alpha$ , IL-6, and IL-18 were examined using the EIA method (Fig. 3). In the AOM-treated mice, not only a high (10 ppm), but also a low (1 ppm) dose of pitavastatin caused a significant decrease in the serum levels of TNF- $\alpha$  (Fig. 3a) and IL-18 (Fig. 3c) in comparison to the control mice ( $P < 0.05$ , respectively). The serum levels of IL-6 (Fig. 3b) were also decreased after treatment with pitavastatin, and this difference was significant after treatment with a high dose of this agent ( $P < 0.05$ ).

**Effects of pitavastatin on the activation of AMPK and on the expression levels of TNF- $\alpha$ , IL-6, IL-18, and COX-2 mRNAs in the colonic mucosa of AOM-injected *db/db* mice.** As shown in Figure 4(a), a western blot analysis demonstrated that treatment with both concentrations of pitavastatin markedly increased the levels of the phosphorylated (i.e. activated) form of AMPK protein in the colonic mucosa of AOM-treated mice. Real-time RT-PCR analyses also revealed that both doses of pitavastatin caused a significant decrease in the expression levels of pro-inflammatory cytokine TNF- $\alpha$  ( $P < 0.05$ , Fig. 4b), IL-6 ( $P < 0.05$ , Fig. 4c), and IL-18 ( $P < 0.01$ , Fig. 4d) mRNAs in the colonic mucosa of AOM-injected mice when compared to those of the untreated control mice. In addition, the mRNA expression levels of COX-2, which is one of the main mediators in the



**Fig. 3.** The effect of pitavastatin on the serum levels of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin (IL)-6, and IL-18 in the azoxymethane (AOM)-treated *db/db* mice (Groups 3 through 5). The serum concentrations of TNF- $\alpha$  (a), IL-6 (b), and IL-18 (c) were measured by an enzyme immunoassay. Values are the mean  $\pm$  SD ( $n = 8$ ). \* $P < 0.05$  vs Group 3.



**Fig. 4.** The effect of pitavastatin on the activation of AMP-activated kinase (AMPK) and on the levels of tumor necrosis factor- $\alpha$  (*TNF- $\alpha$* ), interleukin (*IL-6*, *IL-18*, and cyclooxygenase (*COX-2*) mRNAs in the colonic mucosa of AOM-treated *db/db* mice (Groups 3 through 5). Total proteins were extracted from the scraped colonic mucosa and equivalent amounts of proteins were examined by a Western blot analysis using p-AMPK and AMPK specific antibodies (a). An antibody to GAPDH served as a loading control (a). cDNA was synthesized from the colonic mucosa and real-time RT-PCR was performed using *TNF- $\alpha$*  (b), *IL-6* (c), *IL-18* (d), and *COX-2* (e) specific primers. The expression levels of these genes were normalized to the level of the *GAPDH* gene (b–e). Each experiment was done in triplicate and the average was subsequently calculated. \* $P < 0.05$  and \*\* $P < 0.01$  vs Group 3, respectively.

inflammatory pathway and is certainly involved in CRC development,<sup>(26)</sup> was markedly inhibited by treatment with pitavastatin in comparison to the control mice ( $P < 0.01$ , Fig. 4e).

## Discussion

The results of the present study clearly indicated that pitavastatin effectively inhibits the development of BCAC (Fig. 1c–f), which are putative precursor lesions for CRC,<sup>(21,23)</sup> in male *db/db* obese mice (Table 2). This inhibition was most likely associated with the decrease of pro-inflammatory cytokines, such as *TNF- $\alpha$* , *IL-6*, and *IL-18*, in serum (Fig. 3a–c) as well as in the colonic mucosa (Fig. 4b–d), and the inhibition of proliferation in BCAC (Fig. 2). Pitavastatin also caused a decrease in the expression levels of *COX-2* mRNA (Fig. 4e), which is a critical target for CRC chemoprevention<sup>(26)</sup> in the colonic mucosa of AOM-treated *db/db* mice. These findings are consistent with previous reports that both pitavastatin and simvastatin significantly suppress inflammation-related mouse colon carcinogenesis induced by AOM plus dextran sodium sulfate.<sup>(13,14)</sup> Chronic inflammation, which is caused by obesity, plays a crucial role in the pathogenesis of many chronic diseases, including atherogenesis and carcinogenesis. Furthermore, the beneficial effects of statins on cardiovascular disease have also been linked to their anti-inflammatory properties as well as their lipid-lowering effects.<sup>(27)</sup> Therefore, in addition to reducing the risk for cardiovascular disease, pitavastatin is considered to be effective in preventing cancer development in the colon of obese subjects.

The finding that serum *TNF- $\alpha$*  levels were decreased by pitavastatin (Fig. 3a) is significant because *TNF- $\alpha$*  is an important tumor promoter in inflammation-related carcinogenesis.<sup>(7)</sup> In addition, recent studies have revealed that *TNF- $\alpha$*  lies at the core of the association between obesity and insulin resistance,<sup>(5,28)</sup> which is a key factor for the development of obesity-related CRC, and thus may be a critical target for the prevention of this malignancy.<sup>(1–3,18,19)</sup> Therefore, it was expected that pitavastatin might be able to improve insulin resistance in the present study. However, contrary to our expectations, there was no clear evidence indicating an improvement in insulin resistance by pitavastatin (data not shown). This result might be explained by the lipid solubility of this statin because lipophilic statins may exacerbate insulin resistance, although hydrophilic statins may improve insulin resistance and reduce the risk of diabetes mell-

itus onset.<sup>(29–31)</sup> Therefore, it might be speculated that hydrophilic statins are more effective in suppressing the development of obesity-related CRC by attenuating inflammation and, likely, improving insulin resistance.

Adiponectin, which is secreted by adipose tissue, is regarded as an anti-inflammatory adipocytokine because of its ability to down-regulate the production of *TNF- $\alpha$*  and *IL-6*.<sup>(32)</sup> Circulating levels of adiponectin are decreased in obese individuals, and this phenomenon might be linked to obesity-related carcinogenesis because low adiponectin levels are associated with a higher risk of CRC and an adiponectin-related gene defect has been shown to be involved in this malignancy.<sup>(33–35)</sup> Adiponectin suppresses colonic epithelial proliferation in mice that are fed a high-fat diet.<sup>(36)</sup> In addition, a higher level of serum leptin, which increases *TNF- $\alpha$*  production,<sup>(37)</sup> exerts tumor-promoting effects in obesity- and inflammation-related CRC.<sup>(18,19,38,39)</sup> These reports suggest that abnormalities in the levels of both adiponectin and leptin might be critical targets for the suppression of obesity-related CRC. Therefore, our observations that pitavastatin increases the adiponectin levels and decreases the leptin levels in the serum of AOM-treated *db/db* mice (Table 3) might play a critical role in suppressing obesity-related colorectal carcinogenesis. The effect of pitavastatin on decreasing the serum *TNF- $\alpha$*  levels (Fig. 3a) might be associated with these phenomena because *TNF- $\alpha$*  has been shown to decrease *per se* the expression of adiponectin, while increasing the expression of leptin in adipocytes.<sup>(40,41)</sup>

The present study also showed the first evidence demonstrating that treatment with pitavastatin enhances AMPK activation in the colonic mucosa (Fig. 4a). This might be explained by the elevation of serum adiponectin levels (Table 3) because activation of AMPK, which is a critical monitor of cellular energy status, is involved in the signaling cascade of adiponectin receptors.<sup>(42)</sup> In addition, a recent study has revealed that AMPK controls processes relevant to tumor development and therefore may be a promising target for cancer chemoprevention.<sup>(43)</sup> Pharmacological AMPK activators including metformin, which is used to treat type 2 diabetes mellitus, can inhibit tumor growth of colon cancer xenografts by inducing apoptosis.<sup>(44)</sup> AMP-activated kinase (AMPK) activation is significantly associated with inhibition of *COX-2* expression in CRC cells.<sup>(45)</sup> Moreover, adiponectin inhibits human CRC cell growth by activating AMPK.<sup>(46,47)</sup> Therefore, pitavastatin may exert chemopreventive

properties on obesity-related colorectal carcinogenesis through the elevation of adiponectin (Table 3) and activation of AMPK (Fig. 4a).

Although both BCAC and ACF are regarded as precancerous lesions in the colon, there are clear biological differences between these lesions.<sup>(23,24)</sup> As a result, both histological abnormalities (Fig. 1) and cell proliferative activity (Fig. 2) are significantly increased in BCAC in comparison to ACF.<sup>(48)</sup> Compared to ACF, BCAC are frequently accompanied by Paneth cells,<sup>(48)</sup> which are often present in colon tumors and produce a number of inflammation-related factors including TNF- $\alpha$ .<sup>(49)</sup> In the AOM-induced mouse colon carcinogenesis model, the incidence and location of BCAC are essentially identical to those of colon tumors, while such a coincidence was not observed for ACF.<sup>(50)</sup> In addition, the administration of cholic acid, a tumor promoter for colon cancer, in the diet significantly enhanced both the multiplicity and size of AOM-induced BCAC in the rat, while conversely those of ACF were inhibited by the administration of this agent.<sup>(51)</sup> These findings might provide sufficient evidence that BCAC are more likely to give rise to carcinomas in the colon. Therefore, our findings, namely that pitavastatin markedly inhibited the development of BCAC, appear to be significant when considering the chemoprevention of CRC itself, although this agent did not significantly suppress the development of ACF (Table 2). These findings may be

explained by the biological characteristics of BCAC because these lesions show significant cell proliferative ability and inflammatory responses in comparison to ACF, and can thus be considered a sensitive marker for chemopreventive agents.<sup>(23,24)</sup> Indeed, dietary supplementation with sulindac, a nonsteroidal anti-inflammatory drug, increased the apoptotic index only in BCAC, but not in ACF.<sup>(52)</sup>

In summary, the prevention of CRC by targeting chronic inflammation and adipocytokine imbalance, which is caused by the dysregulation of energy homeostasis, might be a promising strategy for obese people who are at an increased risk for developing CRC. Some types of statins, including pitavastatin, therefore appear to be potentially effective candidates for this purpose because statins can attenuate inflammation while also improving the imbalance of adipocytokines.

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#### Disclosure Statement

The authors have no conflict of interest.

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# Dietary supplementation with branched-chain amino acids suppresses diethylnitrosamine-induced liver tumorigenesis in obese and diabetic C57BL/KsJ-*db/db* mice

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Obesity and related metabolic abnormalities, including insulin resistance, are risk factors for hepatocellular carcinoma in non-alcoholic steatohepatitis as well as in chronic viral hepatitis. Branched-chain amino acids (BCAA), which improve insulin resistance, inhibited obesity-related colon carcinogenesis in a rodent model, and also reduced the incidence of hepatocellular carcinoma in obese patients with liver cirrhosis. In the present study, we determined the effects of BCAA on the development of diethylnitrosamine (DEN)-induced liver tumorigenesis in obese C57BL/KsJ-*db/db* (*db/db*) mice with diabetes mellitus. Male *db/db* mice were given tap water containing 40 ppm DEN for an initial 2 weeks and thereafter they received a basal diet containing 3.0% of BCAA or casein, which served as a nitrogen content-matched control of BCAA, throughout the experiment. Supplementation with BCAA significantly reduced the total number of foci of cellular alteration, a premalignant lesion of the liver, and the expression of insulin-like growth factor (IGF)-1, IGF-2, and IGF-1 receptor in the liver when compared to the casein supplementation. BCAA supplementation for 34 weeks also significantly inhibited both the development of hepatocellular neoplasms and the proliferation of hepatocytes in comparison to the basal diet or casein-fed groups. Supplementation with BCAA improved liver steatosis and fibrosis and inhibited the expression of  $\alpha$ -smooth muscle actin in the DEN-treated *db/db* mice. The serum levels of glucose and leptin decreased by dietary BCAA, whereas the value of the quantitative insulin sensitivity check index increased by this agent, indicating the improvement of insulin resistance and hyperleptinemia. In conclusion, oral BCAA supplementation improves insulin resistance and prevents the development of liver tumorigenesis in obese and diabetic mice. (*Cancer Sci* 2010; 101: 460–467)

Hepatocellular carcinoma is a major health problem worldwide. The development of HCC is frequently associated with chronic inflammation of the liver induced by a persistent infection with the hepatitis B virus or hepatitis C virus.<sup>(1)</sup> The risk of HCC is also elevated in those with metabolic syndrome, also called insulin resistance syndrome, which is commonly associated with obesity and impaired glucose tolerance.<sup>(1–4)</sup> Non-alcoholic fatty liver disease is known to be a hepatic manifestation of the metabolic syndrome. Diabetes mellitus, a condition associated with hyperinsulinemia, has been proposed as a risk factor for both chronic liver disease and HCC through the development of NASH, which is observed in a subset of patients with non-alcoholic fatty liver disease and involves inflammation, cell damage, and/or fibrosis in the liver.<sup>(5–7)</sup> In 1998, Day and James proposed, in their “two hit theory,” that insulin resistance is regarded as a critical factor in the etiology of NASH.<sup>(8)</sup>

C57BL/KsJ-*db/db* (*db/db*) mice are a genetically altered animal model with phenotypes of obesity and diabetes mellitus. A functional defect in the long-form leptin receptor, which plays a significant role in the regulation of food intake and the control of body weight, leads to hyperleptinemia in these mice.<sup>(9)</sup> Because of such obesity, hyperinsulinemia, and hyperleptinemia, the *db/db* mice represent a suitable animal model that uniquely mimics the metabolic syndrome in humans.<sup>(10)</sup> It is also reported that the *db/db* mice are susceptible to chemically induced carcinogenesis in certain tissues. For instance, the *db/db* mice are sensitive to AOM-induced colon carcinogenesis; putative precursor lesions for colorectal cancer are greatly enhanced in *db/db* mice compared to *db/+* or *+/+* mice.<sup>(11)</sup> With respect to the liver, feeding *db/db* mice with a methionine and choline-deficient diet developed accelerated hepatic inflammation and fibrosis, very similar to those seen in human NASH.<sup>(12)</sup>

An improvement of insulin resistance by nutritional or pharmaceutical intervention might therefore be an effective and attractive strategy to inhibit the obesity-related carcinogenesis, as already reported experimentally for the colon.<sup>(13)</sup> Candidate modalities include dietary supplementation with BCAA (leucine, isoleucine, and valine) because BCAA prevents progressive hepatic failure and improves the event-free survival in patients with chronic liver diseases, at least in part, by improving insulin resistance.<sup>(14–16)</sup> In addition, BCAA supplementation has been shown to prevent obesity-related colon carcinogenesis initiated with AOM<sup>(17)</sup> and, furthermore, to reduce the risk of HCC in obese patients with chronic viral liver disease.<sup>(18)</sup> In an obesity-related colon cancer model, the effects of BCAA in inhibiting the development of colonic premalignancies might be associated with improvement of insulin resistance.<sup>(17)</sup> However, whether BCAA prevents obesity-related liver carcinogenesis, and the precise mechanisms of that prevention, have not been explored.

In the present study, we examined the effects of BCAA supplementation on the development of HCC, liver cell adenoma, and FCA in obese and diabetic *db/db* mice initiated with DEN by focusing on the improvement of insulin resistance, liver steatosis, and fibrosis. We also examined whether BCAA supplementation in the diet alters the expression of IGF-1, IGF-2, and IGF-1R in the liver of DEN-treated *db/db* mice. The IGF/IGF-1R axis is closely associated with the development of HCC and might be regarded as a critical target for both HCC treatment and chemoprevention.<sup>(19,20)</sup>

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## Materials and Methods

**Animals, chemicals, and diets.** Four-week-old male *db/db* mice were obtained from Japan SLC (Shizuoka, Japan). All mice received humane care and were maintained at Gifu University Life Science Research Center (Gifu, Japan), according to the Institutional Animal Care Guidelines. DEN was purchased from Sigma Chemical Co. (St. Louis, MO, USA). BCAA and casein were obtained from Ajinomoto Co. (Tokyo, Japan). The BCAA composition (2:1:1.2 = leucine:isoleucine:valine) was set at the clinical dosage that is used for the treatment of decompensated liver cirrhosis in Japan.<sup>(16,18)</sup> The basal diet, CRF-1 (Oriental Yeast Co., Tokyo, Japan), contained 22.4 g of protein (1.65 g leucine, 0.83 g isoleucine, and 1.03 g valine) per 100 g of total volume.

**Experimental procedure.** The experimental protocol was approved by the Institutional Committee of Animal Experiments of Gifu University. At 5 weeks of age, a total of 41 *db/db* mice were divided into three groups. All the mice in Group 1 ( $n = 11$ ), Group 2 ( $n = 15$ ), and Group 3 ( $n = 15$ ) were given tap water containing 40 ppm DEN for the initial 2 weeks. After treatment with DEN, Group 3 was given the CRF-1 supplemented with 3.0% BCAA (w/w) through to the end of experiment, whereas mice in Group 2 were given the basal diet supplemented with 3.0% casein (w/w) and served as a nitrogen content-matched control for the BCAA-treated group. Group 1 was given the CRF-1 diet throughout the experiment. In order to examine the effect of BCAA on the development of FCA in early phase, four mice each in groups 2 and 3 were starved for 6 h and killed by CO<sub>2</sub> asphyxiation at 23 weeks of age (after 16 weeks supplementation with BCAA or casein). At 41 weeks of age (after 34 weeks supplementation with the experimental diet), all remaining animals (total 33 mice) were killed to determine the development of HCC, liver cell adenoma, and FCA.

**Histopathology and immunohistochemical analyses for  $\alpha$ -SMA and PCNA.** After the mice were killed, the livers were immediately removed and macroscopically inspected for the presence of neoplasms. Maximum sagittal sections of each lobe (six lobes) were used for histological examination. The tissue specimens were fixed in 10% buffered formaldehyde then embedded in paraffin. Serial sections (3–4  $\mu$ m thick) were cut from the tissue blocks and stained with H&E for histopathology or Azan stain to observe liver fibrosis. The liver neoplasms (HCC and liver cell adenoma) and FCA were diagnosed according to criteria described previously.<sup>(21)</sup> The multiplicity of FCA was assessed on the per area basis (per cm<sup>2</sup>).

Immunohistochemistry of  $\alpha$ -SMA, an indicator of HSC activation, was carried out using a primary anti- $\alpha$ -SMA antibody (Dako, Glostrup, Denmark).<sup>(22)</sup> Immunohistochemistry of PCNA, a G<sub>1</sub>-to-S phase marker, was carried out to estimate the cell proliferative activity of the hepatocyte using a primary anti-PCNA antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA).<sup>(23)</sup> PCNA-positive nuclei in the hepatocytes were counted and expressed as the percentage of the total number of hepatocyte nuclei. The PCNA-labeling index (%) was determined by counting at least 500 hepatocytes in each section (total of 3000 hepatocytes per mouse).

**Clinical chemistry.** After the mice were killed, blood samples were collected from inferior vena cava to determine the serum concentrations of ALT, glucose, insulin, leptin, and BCAA. The levels of serum glucose, insulin, and BCAA were assayed as described previously.<sup>(24,25)</sup> The serum leptin level was determined by ELISA (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocol.<sup>(17)</sup> The serum ALT activity was measured with a standard clinical automatic analyzer (type 726; Hitachi, Tokyo, Japan). Insulin resistance was estimated by QUICKI as follows: QUICKI =  $1/[\log(I_0) + \log(G_0)]$ , where  $I_0$  is the fasting insulin and  $G_0$  is the fasting glucose, which correlates with the glucose clamp method.<sup>(26)</sup>

**Hepatic lipid analysis.** To visualize intrahepatic lipids, Sudan III stain was carried out with frozen sections using the standard procedure. The hepatic lipids were also extracted from the frozen livers. Approximately 200 mg of liver was homogenized and the lipids were then extracted using chloroform:methanol (2:1 v/v) solution, as described by Folch.<sup>(27)</sup> The levels of triglyceride in the liver were measured using the triglyceride E-test kit (Wako Pure Chemical Co., Osaka, Japan) according to the manufacturer's protocol.

**Hepatic hydroxyproline analysis.** Hepatic hydroxyproline content was quantified colorimetrically in duplicate samples from approximately 200 mg wet-weight of liver tissue, as previously described.<sup>(22)</sup> The hydroxyproline contents were expressed as  $\mu$ mol/g wet liver.

**Protein extraction and Western blot analysis.** Equivalent amounts of protein lysates (30  $\mu$ g/lane) from the liver of experimental mice were subjected to a Western blot analysis of  $\alpha$ -SMA (Dako), as described previously.<sup>(22,23)</sup> An antibody to GAPDH (Chemicon International, Temecula, CA, USA) served as a loading control. The intensities of the blots were quantified with NIH image software version 1.62.

**RNA extraction and quantitative real-time RT-PCR analysis.** A quantitative real-time RT-PCR analysis was carried out as described previously.<sup>(28)</sup> Total RNA was isolated from the liver of the mice using the RNAqueous-4PCR kit (Ambion Applied Biosystems, Austin, TX, USA). The cDNA was synthesized from 0.2  $\mu$ g total RNA using the SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). The primers used for the amplification of *IGF-1*, *IGF-2*, and *IGF-1R* specific genes were as follows: *IGF-1* forward, 5'-CTGGACCAGAGACCCCTTGC-3' and reverse, 5'-GGACGGGGACTTCTGAGTCTT-3'; *IGF-2* forward, 5'-GTGCTGCATCGCTGCTTAC-3' and reverse, 5'-ACGTCCTCTCGGACTTGG-3'; and *IGF-1R* forward, 5'-GTGGGGGCTCGTGTTCCTC-3' and reverse, 5'-GATCACCGTGCAGTTTTCCA-3'. Real-time PCR was done in a LightCycler (Roche Diagnostics Co., Indianapolis, IN, USA) with SYBR Premix Ex Taq (TaKaRa Bio, Shiga, Japan). The expression levels of the *IGF-1*, *IGF-2*, and *IGF-1R* genes were normalized to the  $\beta$ -actin gene expression level.<sup>(28)</sup>

**Statistical analysis.** The results are presented as the mean  $\pm$  SD, and they were analyzed using the GraphPad Instat software program version 3.05 (GraphPad Software, San Diego, CA, USA) for Macintosh. Differences among the groups were analyzed by either one-way ANOVA or, as required, by two-way ANOVA. When ANOVA showed a statistically significant effect ( $P < 0.05$ ), comparisons of each experimental group with the control group were then made using Dunnett's test, which corrects for multiple comparisons. The differences were considered to be significant when the two-sided  $P$  value was  $< 0.05$ .

## Results

**General observations.** As shown in Table 1, there were no significant differences in the body, liver, kidney, or fat (white adipose tissue of the periorchis and retroperitoneum) weights among the groups at the end of the study. Male *db/db* mice well-tolerated the treatment with DEN together with casein or BCAA. The body weight gains did not differ significantly among the groups during the experiment (data not shown). A histopathological examination suggested the absence of toxicity of BCAA in important organs, including liver, kidney, and spleen (data not shown). In addition, no clinical signs indicating the toxicity of BCAA were observed in the mice during the experiment.

**Incidence and multiplicity of DEN-induced liver neoplasms and FCA in *db/db* mice.** Macroscopically, nodular lesions (Fig. 1a) were observed in the livers of experimental mice at the termination of the study (41 weeks of age). Histopathologically, these lesions were liver cell adenoma (Fig. 1b) or HCC (Fig. 1c).

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

Group no.	Diet	No. of mice	Weight (g) (mean $\pm$ SD)			
			Body	Liver	Kidney	Fat†
1	CRF-1	11	73.0 $\pm$ 9.2	4.4 $\pm$ 0.9	0.9 $\pm$ 1.0	7.8 $\pm$ 2.2
2	Casein	11	66.0 $\pm$ 12.0	3.8 $\pm$ 1.2	0.6 $\pm$ 0.2	5.4 $\pm$ 1.0
3	BCAA	11	68.2 $\pm$ 12.4	3.4 $\pm$ 1.3	0.6 $\pm$ 0.1	6.2 $\pm$ 1.4

†White adipose tissue of the periorchis and retroperitoneum.

FCA (Fig. 1d) also developed in the liver of experimental mice. Simultaneously, we put supplemental groups to support that *db/db* mice are actually susceptible to DEN-induced liver tumorigenesis (data not shown; see Supporting Information Table S1) and found no neoplasms in C57B6 or C57BL/KsJ-*+/+* mice, genetic controls for *db/db* mice, regardless of DEN treatment. No tumors developed in the CRF-1-fed and DEN-untreated *db/db* mice.

**Effects of BCAA supplementation on DEN-induced liver tumorigenesis in *db/db* mice.** The incidence and multiplicity of liver neoplasms (adenoma plus HCC) and FCA at 41 weeks of age are summarized in Table 2. Compared with the CRF-1-fed mice (Group 1), dietary supplementation with BCAA (Group 3) significantly inhibited the incidence ( $P < 0.05$ ) of adenoma. BCAA supplementation also reduced the incidence ( $P < 0.05$ ) and multiplicity of adenoma ( $P < 0.01$ ) compared to the casein-supplementation mice (Group 2). HCC was developed in the CRF-1-fed (9%) and casein-supplementation mice (27%), but not in the mice supplemented with BCAA (0%), and the multiplicity of total liver neoplasms was significantly inhibited by supplementation with BCAA when compared to CRF-1-fed ( $P < 0.05$ ) or Casein-supplementation mice ( $P < 0.01$ ), respectively. The number of FCA, which were developed in all experimental mice, was also significantly decreased by supplementation with BCAA when compared to CRF-1-fed ( $P < 0.05$ ) or casein-supplementation mice ( $P < 0.001$ ), respectively.

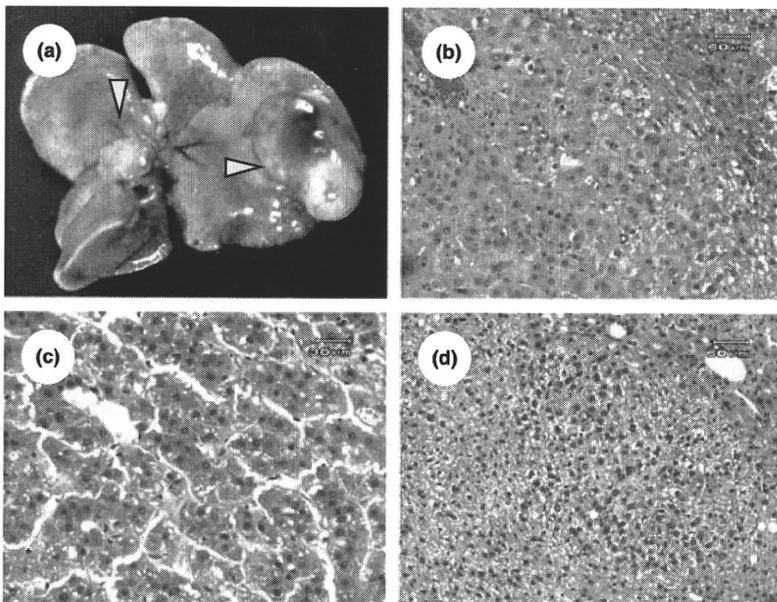
**Effects of BCAA supplementation on the expression levels of *IGF-1*, *IGF-2*, and *IGF-1R* mRNAs in the liver of DEN-treated *db/db* mice.** When the mice were killed at 23 weeks of age, the development of FCA was also significantly inhibited by dietary supplementation with BCAA compared with casein-supplemented

mice ( $P < 0.01$ ) (Fig. 2a). In addition, semiquantitative RT-PCR analyses showed that there was a significant decrease in the expression level of *IGF-1* ( $P < 0.05$ ), *IGF-2* ( $P < 0.05$ ), and *IGF-1R* mRNAs ( $P < 0.05$ ) in the livers of the mice supplemented with BCAA when compared to that of the livers in casein-supplemented mice (Figs 2b–d). These findings suggest that BCAA supplementation prevents the development of FCA, at least in part, by inhibiting the expression of the IGF/IGF-1R axis.

**Effects of BCAA supplementation on serum levels of BCAA, ALT, and leptin in DEN-treated *db/db* mice.** BCAA supplementation caused a significant increase in the serum levels of BCAA compared to the CRF-1-fed ( $P < 0.05$ ) and casein-supplemented mice ( $P < 0.05$ ) (Fig. 3a). These findings suggest that supplementation with 3.0% BCAA is sufficient to raise the serum concentration of BCAA. The serum ALT levels markedly increased in the *db/db* mice when compared to the genetic control mice (data not shown; see Supporting Information Table S1). However, BCAA supplementation significantly decreased this value in comparison to the CRF-1-fed ( $P < 0.01$ ) and casein-supplemented mice ( $P < 0.001$ ) (Fig. 3b), thus indicating an improvement of liver damage. In addition, the mice supplemented with BCAA showed a decrease in the serum levels of leptin compared with the CRF-1-fed ( $P < 0.001$ ) and casein-supplemented mice ( $P < 0.001$ ) (Fig. 3c).

**Effects of BCAA supplementation on the hepatic steatosis in DEN-treated *db/db* mice.** Examination of Sudan III stained sections revealed that there was a marked macrovesicular steatosis in the DEN-treated *db/db* mice, which were fed CRF-1 or casein, but BCAA supplementation significantly improved the accumulation of the lipid in the liver (Fig. 4a). The histological findings were consistent with the results of the measurement of liver triglyceride contents; the levels of triglyceride in the liver of DEN-treated *db/db* mice were significantly decreased by the supplementation with BCAA compared to those in the CRF-1-fed ( $P < 0.001$ ) and casein-supplementation groups ( $P < 0.01$ ) (Fig. 4b).

**Effects of BCAA supplementation on liver fibrosis in DEN-treated *db/db* mice.** As shown in Figure 5a, examination of Azan-stained sections indicated that DEN-treated *db/db* mice of CRF-1-fed and casein-supplemented groups showed the development of peri-central venous and peri-cellular fibrosis. However, supplementation with BCAA yielded an improvement in liver fibrosis (Fig. 5a). Similar findings were also observed in

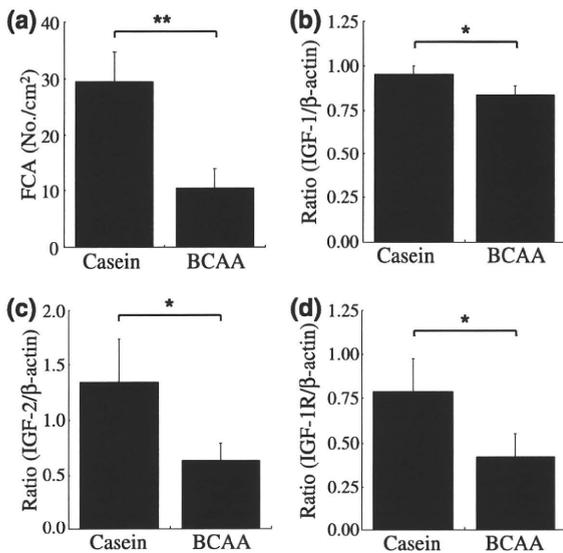


**Fig. 1. Macroscopic (a) and microscopic (b–d) analyses of liver neoplasms in diethylnitrosamine-treated *db/db* mice. (a) Macroscopically, white tumors (hepatocellular carcinoma; indicated by arrowheads) were detected in the liver of diethylnitrosamine-treated C57BL/KsJ-*db/db* mice. (b–d) Paraffin-embedded sections were stained with H&E. Representative photomicrographs show adenoma (b), hepatocellular carcinoma (c), and foci of cellular alteration (d) in liver of experimental mice.**

**Table 2.** Incidence and multiplicity of hepatic neoplasms and foci of cellular alteration (FCA) in obese diabetic C57BL/KsJ-*db/db* mice fed basal (CRF-1), casein-supplemented, or branched-chain amino acid (BCAA)-supplemented diets

Group no.	Diet	No. of mice	Incidence (%)		Multiplicity (no. of neoplasms/mouse) (mean ± SD)			FCA (No./cm <sup>2</sup> ) (mean ± SD)
			Adenoma	HCC	Total	Adenoma	HCC	
1	CRF-1	11	7/11 (64)	1/11 (9)	1.0 ± 1.1	0.9 ± 1.1	0.1 ± 0.3	14.4 ± 4.4
2	Casein	11	8/11 (73)	3/11 (27)	1.7 ± 1.3	1.5 ± 1.1	0.3 ± 0.5	19.1 ± 5.7
3	BCAA	11	2/11 (18)*, **	0/11 (0)	0.2 ± 0.4*, ***	0.2 ± 0.4***	0	9.6 ± 5.1*, ****

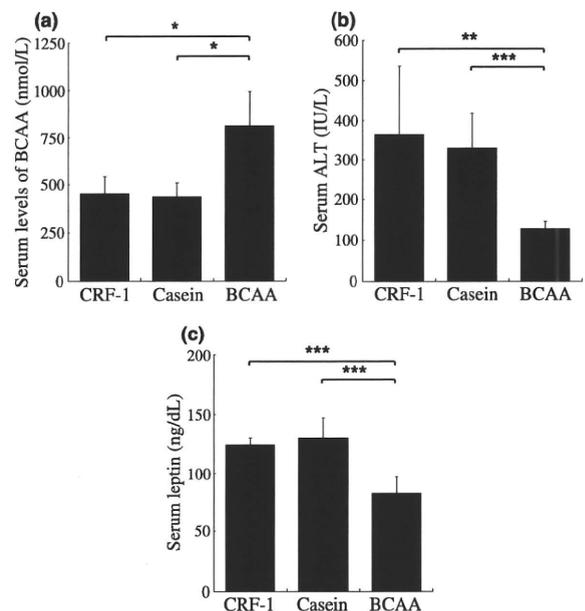
\* $P < 0.05$ , significantly different from Group 1; \*\* $P < 0.05$ , significantly different from Group 2; \*\*\* $P < 0.01$ , significantly different from Group 2; \*\*\*\* $P < 0.001$ , significantly different from Group 2. HCC, hepatocellular carcinoma.



**Fig. 2.** Effect of branched-chain amino acid (BCAA) supplementation on the development of foci of cellular alteration (FCA) and on the expression of insulin-like growth factor (IGF)-1, IGF-2 and IGF-1 receptor (IGF-1R) mRNAs in the liver of diethylnitrosamine-treated C57BL/KsJ-*db/db* mice. Livers were excised from treated mice supplemented with casein or BCAA for 16 weeks. (a) Paraffin-embedded liver sections were stained with H&E and the total numbers of FCA were counted. Values are the means ± SD ( $n = 4$ ). (b–d) Total RNA was isolated from the removed liver and the expression of *IGF-1* (b), *IGF-2* (c), and *IGF-1R* (d) genes were examined by quantitative real-time RT-PCR. The expression of each gene was normalized to  $\beta$ -actin expression. Each experiment was done in triplicate. \* $P < 0.05$ ; \*\* $P < 0.01$ .

the measurement of liver hydroxyproline contents, a useful marker of hepatic fibrosis,<sup>(22)</sup> when compared to CRF-1 feeding ( $P < 0.05$ ) and casein supplementation ( $P < 0.01$ ), BCAA supplementation caused a significant decrease in the amounts of hydroxyproline in the liver of DEN-treated *db/db* mice (Fig. 5b). In addition, both the immunohistochemical (Fig. 6a) and Western blot analyses (Fig. 6b) showed the expression levels of  $\alpha$ -SMA in the liver to be elevated in the CRF-1-fed and casein-supplemented mice, whereas supplementation with BCAA significantly decreased the expression of this protein ( $P < 0.05$  and  $P < 0.01$ , respectively).

**Effects of BCAA supplementation on insulin resistance and serum level of glucose in DEN-treated *db/dbdb* mice.** Insulin resistance plays a critical role in obesity-related HCC development.<sup>(1–4)</sup> Therefore, the effects of BCAA supplementation on the value of QUICKI and the serum levels of glucose were examined in DEN-treated *db/db* mice. As shown in Figure 7a, supple-



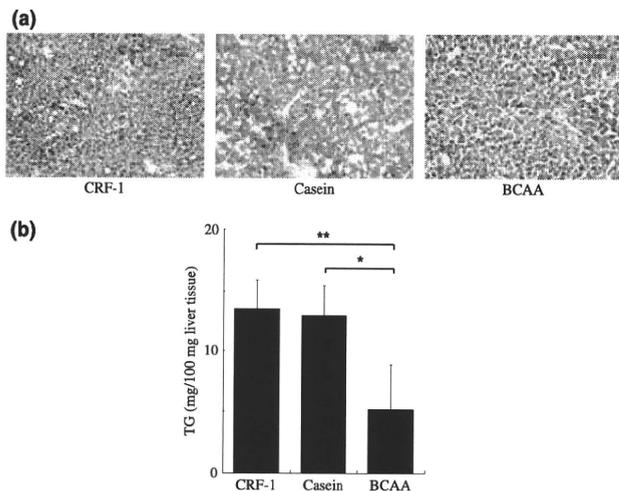
**Fig. 3.** Effect of branched-chain amino acid (BCAA) supplementation on the serum levels of BCAA, alanine aminotransferase (ALT), and leptin in diethylnitrosamine-treated C57BL/KsJ-*db/db* mice. After mice were killed, blood samples were collected and the serum levels of BCAA (a), ALT (b), and leptin (c) were then assayed. Values are the means ± SD ( $n = 8$ ). \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

mentation with BCAA caused a significant increase in the value of QUICKI compared to the CRF-1-fed ( $P < 0.01$ ) and casein-supplemented mice ( $P < 0.01$ ), thus indicating an improvement of insulin resistance. The serum glucose level also decreased after the supplementation with BCAA compared to CRF-1-fed ( $P < 0.001$ ) and casein-supplementation ( $P < 0.01$ ) (Fig. 7b).

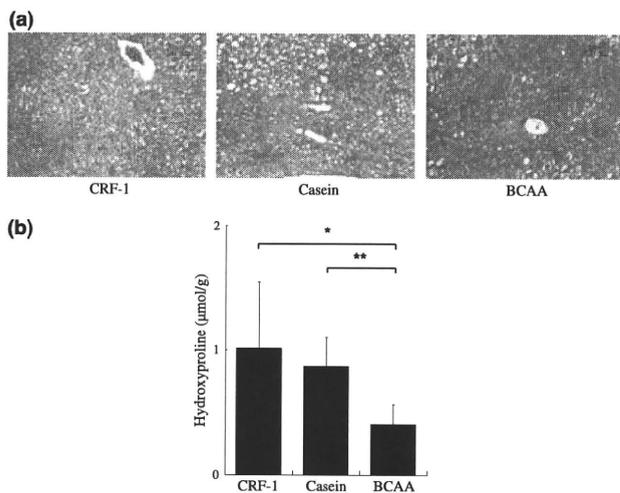
**Effects of BCAA supplementation on cell proliferative activity in liver of DEN-treated *db/db* mice.** The PCNA-labeling index of non-lesional hepatocytes in DEN-treated *db/db* mice was determined based on the findings of PCNA-immunohistochemical sections (Fig. 8a). As illustrated in Figure 8b, the mean PCNA-labeling index in the BCAA-supplemented mice was significantly lower than that of the CRF-1-fed ( $P < 0.01$ ) and casein-supplemented mice ( $P < 0.05$ ), thus indicating that BCAA supplementation significantly inhibited cell proliferation in the liver of DEN-treated *db/db* mice.

## Discussion

Recent studies have shown that obesity and diabetes mellitus are risk factors for HCC through the development of NASH.<sup>(5–7)</sup> The



**Fig. 4.** Effect of branched-chain amino acid (BCAA) supplementation on hepatic steatosis in diethylnitrosamine-treated C57BL/KsJ-*db/db* mice. (a) Frozen sections of basal diet (CRF-1)-fed, casein-supplemented, or BCAA-supplemented treated mice were stained with Sudan III stain to show steatosis. (b) Hepatic lipids were extracted from the frozen livers and the levels of triglyceride were then measured. Values are the means  $\pm$  SD ( $n = 8$ ). \* $P < 0.01$ ; \*\* $P < 0.001$ .



**Fig. 5.** Effect of branched-chain amino acid (BCAA) supplementation on hepatic fibrosis in diethylnitrosamine-treated C57BL/KsJ-*db/db* mice. (a) Paraffin-embedded sections of basal diet (CRF-1)-fed, casein-supplemented, or BCAA-supplemented treated mice were stained with Azan stain to show fibrosis. (b) Hepatic hydroxyproline contents were quantified colorimetrically. Values are the means  $\pm$  SD ( $n = 8$ ). \* $P < 0.05$ ; \*\* $P < 0.01$ .

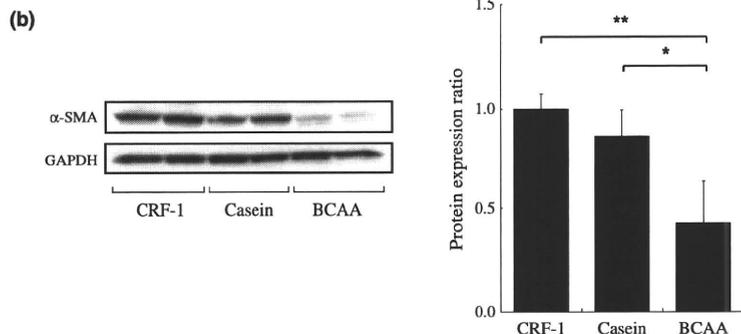
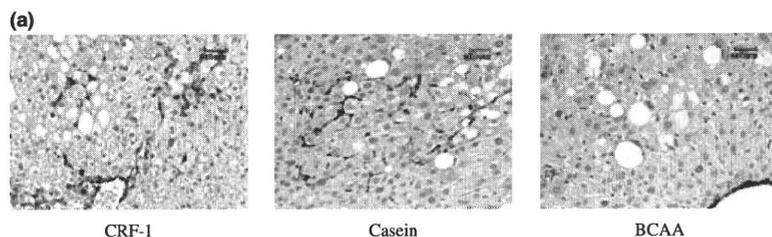
present study clearly indicated that *db/db* mice, which develop obesity and severe diabetes mellitus, easily developed steatosis-related liver neoplasms by treatment with liver carcinogen DEN (Table 2 and Fig. 1), whereas background C57B6 or C57BL/KsJ-+/+ mice did not. Furthermore, this study showed that dietary supplementation with BCAA effectively decreased the serum levels of ALT (Fig. 3b), which increase due to severe steatosis (Fig. 4a) and fibrosis (Fig. 5a), and inhibited the development of liver neoplasms (Table 2) in DEN-treated *db/db* mice. A clinical trial recently indicated that dietary supplementation with BCAA can reduce the risk of HCC in cirrhotic patients who

are obese.<sup>(18)</sup> How can BCAA exert chemopreventive effects on obesity-related HCC? Presumably, the improvement of insulin resistance by BCAA (Fig. 7a) plays a critical role in this beneficial effect because, in addition to the role of insulin in glucose uptake and glycogen biosynthesis in liver and skeletal muscle, insulin has oncogenic properties on HCC cells, including the stimulation of cell growth and induction of anti-apoptotic activity.<sup>(29,30)</sup> These reports, therefore, suggest the possibility that BCAA inhibits the excessive cell proliferation in the whole liver of DEN-treated *db/db* mice (Fig. 8) by improving insulin resistance (Fig. 7a). Recent studies have also revealed that BCAA improves glucose tolerance by modulating the insulin-independent glucose uptake into skeletal muscle.<sup>(31,32)</sup> Isoleucine increased muscle glucose uptake and depressed gluconeogenesis in the liver without causing significant elevation of the plasma insulin level, thereby leading to the hypoglycemic effect in a rodent model.<sup>(33)</sup> Both improved insulin resistance and glucose tolerance by BCAA have also been indicated in clinical trials.<sup>(14,34)</sup>

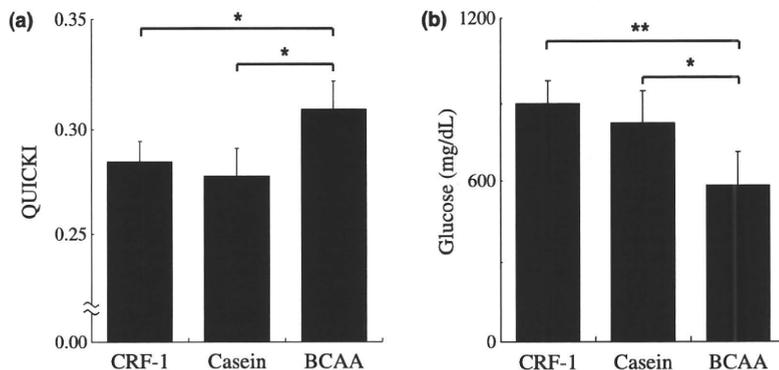
In addition to the improvement of insulin resistance (Fig. 7a), the present study also indicated that dietary supplementation with BCAA significantly decreased the expression levels of *IGF-1*, *IGF-2*, and *IGF-1R* mRNAs in the liver of DEN-treated *db/db* mice (Figs 2b–d). These findings seem to be significant because abnormal activation of the IGF/IGF-1R axis, which is caused by insulin resistance, is involved in the development of HCC and, therefore, might be a critical target to prevent this malignancy.<sup>(19,20)</sup> These findings are also consistent with those of a previous report that showed BCAA supplementation decreased the serum levels of both IGF-1 and IGF-2 while also inhibiting the expression of IGF-1R on the colonic mucosa, thereby preventing the development of AOM-induced colonic neoplastic lesions in *db/db* mice.<sup>(17)</sup> This previous report,<sup>(17)</sup> together with our present findings (Fig. 2), suggest the possibility that the inhibition of IGF/IGF-1R activation is one of the critical mechanisms to suppress obesity-related tumorigenesis in specific organs, such as the colon and liver, and BCAA might be able to exert its chemopreventive effect on obesity-associated carcinogenesis by targeting this axis.

Insulin stimulates glucose uptake and triglyceride biosynthesis, which are stored in adipose tissue. The improvement of insulin resistance by BCAA, therefore, inhibits the release of free fatty acid from adipose tissue, improves hypertriglyceridemia, and thus resulted in improvement of hepatic steatosis in the present study (Fig. 4). Ectopic triglyceride accumulation in the liver is directly responsible for the development of insulin resistance.<sup>(35)</sup> In addition, several studies support the concept that hepatic steatosis promotes the development of HCC.<sup>(36)</sup> For instance, HCV core protein gene transgenic mice, a model for HCV-related hepatocarcinogenesis,<sup>(37)</sup> show marked hepatic steatosis and insulin resistance.<sup>(38,39)</sup> Hepatic steatosis is a major accelerating factor of hepatocarcinogenesis in chronic HCV infected patients.<sup>(40)</sup> In addition, a significant relationship has also been reported between steatosis and hepatic fibrosis, a potent risk factor for HCC development.<sup>(36)</sup> Therefore, the reduction of hepatic lipid accumulation might be an effective strategy for HCC chemoprevention. The improvement of hepatic steatosis (Fig. 4) and fibrosis (Fig. 5) by BCAA is thus considered to be advantageous to accomplish this objective.

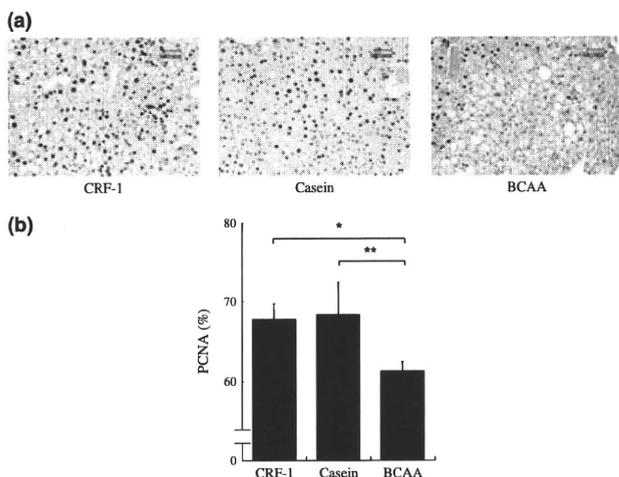
There are two study limitations that might suggest additional investigations. The first is that the incidence of HCC itself was not very high in the present study (Table 2) because the duration of the experiments (41 weeks) might have been sufficient to develop adenoma but not HCC. Therefore, future study should recruit longer-term experiments to see that DEN-treated *db/db* mice develop HCC more frequently. The second is that, although our model seems to be useful to elucidate the pathogenesis underlying NASH-associated HCC, there is one difference between the liver of *db/db* mice and human NASH, as hepatic fibrosis was



**Fig. 6.** Effect of branched-chain amino acid (BCAA) supplementation on the expression of  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) in diethylnitrosamine-treated C57BL/KsJ-*db/db* mice. (a) Immunohistochemical expression of  $\alpha$ -SMA in the liver of basal diet (CRF-1)-fed, casein-supplemented, or BCAA-supplemented treated mice. (b) Total protein was extracted from the liver of experimental mice and the expression of  $\alpha$ -SMA protein was examined by Western blot analysis. An antibody to GAPDH served as a loading control. Repeat Western blots gave similar results. The results obtained were quantitated by densitometry and are shown in the right-hand panels. Values are the means  $\pm$  SD ( $n = 5$ ). \* $P < 0.05$ ; \*\* $P < 0.01$ .



**Fig. 7.** Effect of branched-chain amino acid (BCAA) supplementation on insulin sensitivity and the serum level of glucose in diethylnitrosamine-treated C57BL/KsJ-*db/db* mice fed basal diet (CRF-1), or supplemented with casein or BCAA. (a) The value of the quantitative insulin sensitivity check index (QUICKI), was calculated to evaluate the insulin sensitivity. (b) The serum concentration of glucose was measured by the hexokinase method. Values are the means  $\pm$  SD ( $n = 8$ ). \* $P < 0.01$ ; \*\* $P < 0.001$ .



**Fig. 8.** Effect of branched-chain amino acid (BCAA) supplementation on hepatic cell proliferation in diethylnitrosamine-treated C57BL/KsJ-*db/db* mice. (a) Immunohistochemical expression of proliferating cell nuclear antigen (PCNA) in the liver of basal diet (CRF-1)-fed, or casein- or BCAA-supplemented treated mice. (b) PCNA-labeling index in non-lesional hepatocytes was determined by counting the PCNA-positive nuclei in the hepatocytes. \* $P < 0.05$ ; \*\* $P < 0.01$ .

severe but did not reach liver cirrhosis at the end point of this experiment (Fig. 5a). This might be explained by a functional defect in the long-form leptin receptor because leptin exerts a pro-fibrogenic activity in the injured liver.<sup>(41,42)</sup> However, BCAA supplementation significantly decreased the serum levels of leptin (Fig. 3c), inhibited the development of liver fibrosis (Fig. 5), and suppressed the expression of  $\alpha$ -SMA (Fig. 6), thus indicating the inhibition of HSC activation. These findings seem to be significant because activated HSCs are a major cellular source of collagen in the injured liver and thus may be a critical target for inhibiting the development of liver fibrosis.<sup>(43)</sup> Therefore, BCAA supplementation prevents the development of hepatic fibrosis, at least in part, by inhibiting the HSC activation (Fig. 6). In addition, a previous study also indicated that supplementation with BCAA effectively suppressed the hyperleptinemia in *db/db* mice with colonic carcinogenesis model.<sup>(17)</sup> These findings suggest that leptin is also one of the critical targets of BCAA in obese mice. Future studies would be important to evaluate whether BCAA could also prevent the development of liver fibrosis using a more aggressive fibrotic model, such as methionine and choline-deficient diet-fed *db/db* mice, known to be a good model of progressive NASH.<sup>(44)</sup>

Finally, it should be emphasized again that, in a recent study, BCAA supplementation in the basal diet was shown to improve insulin resistance, thereby preventing the development of colonic

pre-malignancies in an obesity-related colon cancer model.<sup>(17)</sup> Both obesity and insulin resistance are strongly associated with the development of not only HCC, but also colorectal cancer.<sup>(45)</sup> These previous reports, therefore, further strengthen our conclusion that the prevention of HCC by targeting the dysregulation of energy homeostasis, particularly an increased insulin resistance, might be a promising strategy for obese people who are at increased risk for developing HCC. BCAA appears to be a potentially effective and critical candidate for this purpose because it can improve insulin resistance (Fig. 7a), hepatic steatosis (Fig. 4), and fibrosis (Fig. 5) in obese and diabetic *db/db* mice.

In conclusion, BCAA might therefore represent a new effective strategy for chemoprevention against HCC, especially in obese people. Among the beneficial effects of BCAA shown in this study, the improvement of insulin resistance might play a crucial role to prevent the development of obesity-related liver tumorigenesis because the state of insulin resistance is closely associated with the activation of the IGF/IGF-1R axis, the development of hepatic steatosis and fibrosis.<sup>(5-7)</sup> In addition, a recent study revealed that BCAA supplementation also suppressed hepatic neovascularization in insulin-resistance-based hepatocarcinogenesis in obese rats.<sup>(46)</sup>

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## Abbreviations

$\alpha$ -SMA	$\alpha$ -smooth muscle actin
ALT	alanine aminotransferase
AOM	azoxymethane
BCAA	branched-chain amino acids
DEN	diethylnitrosamine
FCA	foci of cellular alteration
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HSC	hepatic stellate cell
IGF	insulin-like growth factor
IGF-1R	insulin-like growth factor-1 receptor
NASH	non-alcoholic steatohepatitis
PCNA	proliferating cell nuclear antigen
QUICKI	quantitative insulin sensitivity check index

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Table S1.** Incidence and multiplicity of hepatic neoplasms and FCA and serum levels of ALT in db/db, +/- and B6 mice.

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# Insulin resistance raises the risk for recurrence of stage I hepatocellular carcinoma after curative radiofrequency ablation in hepatitis C virus-positive patients: A prospective, case series study

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**Aim:** Several studies have reported that insulin resistance raises the risk of primary hepatocellular carcinoma (HCC). We conducted a prospective, case series study to test the impact of insulin resistance on the recurrence after curative radiofrequency ablation (RFA) of stage I HCC in HCV-positive patients.

**Methods:** From January 2006 to December 2007, 226 consecutive patients underwent treatment for primary HCC at our institutions, including 37 stage I cases. Among them, 33 were HCV-positive, and three, six and 24 received curative surgery, transarterial chemoembolization or RFA, respectively. In the 24 patients treated with RFA, recurrence-free survival was analyzed using the Kaplan–Meier method. The factors contributing to recurrence of HCC were subjected to univariate and multivariate analyses using the Cox proportional hazards model. Insulin resistance was estimated by the Homeostatic Model Assessment of Insulin Resistance (HOMA-IR).

**Results:** Kaplan–Meier analysis showed that the recurrence-free survival was lower in patients with higher HOMA-IR (>2.3,

$P = 0.0252$ ) or with lower serum albumin level (<3.3 g/dL,  $P = 0.0004$ ). In the univariate analysis, HOMA-IR ( $P = 0.0420$ ) and albumin ( $P = 0.0036$ ) were significantly associated with recurrence of HCC. Multivariate analysis revealed albumin (odds ratio = 0.01, 95% confidence interval = 0.0002–0.015,  $P = 0.0001$ ) and HOMA-IR (odds ratio = 3.85, 95% confidence interval = 1.57–14.2,  $P = 0.0015$ ) to be independent predictors for recurrence of HCC.

**Conclusion:** Serum albumin level and HOMA-IR were independent risk factors for recurrence of stage I HCC after curative RFA in HCV-positive patients. Patients with these factors require closer surveillance.

**Key words:** hepatitis C virus, hepatocellular carcinoma, Homeostatic Model Assessment of Insulin Resistance, insulin resistance, radiofrequency ablation, recurrence

## INTRODUCTION

HEPATOCELLULAR CARCINOMA (HCC) is prevalent worldwide, especially in Africa and the Western Pacific Region. HCC is the third most common cause of cancer death in men and the fifth most common in women; every year, more than 600 000 people die from this disease ([www.who.int/whosis/](http://www.who.int/whosis/)).

Risk factors for the development of primary HCC include viral infection such as hepatitis B virus (HBV)

and hepatitis C virus (HCV), alcohol consumption, aflatoxin and immune-related hepatitis.<sup>1</sup> Regarding risk factors for recurrence, several studies have suggested male sex, presence of cirrhosis, high  $\alpha$ -fetoprotein (AFP), large tumor foci, multiplicity of tumors, pathologically high-grade atypia of tumor cells and presence of portal venous invasion of tumor.<sup>2–6</sup> Recently, several epidemiological studies have revealed a close association between diabetes mellitus (DM) and HCC. Wideroff *et al.*<sup>7</sup> described the standardized incidence ratios in Denmark for primary liver cancer in subjects with DM compared with the general population as 4.0 (95% confidence interval [CI] = 3.5–4.6) and 2.1 (95% CI = 1.6–2.7) for men and women, respectively. El-Serag *et al.*<sup>8</sup> reported that DM increased the risk of chronic non-alcoholic liver disease and HCC in male patients without concomitant

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liver disease in the USA. Furthermore, patients with chronic hepatitis and cirrhosis tend to experience complications with DM or to show insulin resistance.<sup>9</sup> This is particularly the case for patients with HCV infection and non-alcoholic fatty liver disease (NAFLD), including its most severe form, non-alcoholic steatohepatitis (NASH), which can lead directly to HCC.<sup>10–12</sup> The HCV core protein induced insulin resistance by increasing tumor necrosis factor- $\alpha$  which disrupts tyrosine phosphorylation of insulin receptor substrate-1.<sup>13</sup> Thus, DM including insulin resistance seems to be closely associated with various liver diseases that can lead to HCC, although the impact of insulin resistance on the recurrence of HCC has not been evaluated.

In this study, to identify the impact of insulin resistance on recurrence after initial curative treatment for HCC, we designed a prospective, case series analysis to examine recurrence-free survival in consecutive patients with stage I HCC, stratified by Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) level, which is commonly used for measuring insulin resistance.<sup>14,15</sup> In particular, we focused on HCV-positive patients who were treated with radiofrequency ablation (RFA).

## METHODS

### Patients

FROM JANUARY 2006 to December 2007, 226 primary HCC patients underwent initial treatment at our institutions, and 199 of them were followed to the end of this study (April 2008). Among them, we had 37 consecutive patients with stage I HCC that met all the criteria: a single tumor of 2 cm or less diameter, with no vascular invasion, no lymph-node invasion and no distant metastasis.<sup>16</sup>

Hepatocellular carcinoma nodules were detected by imaging modalities including abdominal ultrasonography, dynamic computed tomography (CT), dynamic magnetic resonance imaging (MRI) and abdominal arteriography. Diagnosis of HCC was made from a typical hypervascular tumor stain on angiography and typical dynamic-study findings of enhanced staining in the early phase and attenuation in the delayed phase. Etiologies for HCC were HCV in 33 patients, HBV in two and others in two.

### Treatment, follow up and determination of recurrence

Three patients were treated with surgical resection, six with transarterial chemoembolization (TACE) and 28

with RFA. Among them, we only recruited those who were positive for HCV and treated with RFA ( $n = 24$ ). Therapeutic effect was judged to be curative using dynamic CT or MRI with total disappearance of imaging characteristics of HCC as described above.

Patients were thereafter followed on an out-patient basis using serum tumor markers such as AFP and protein induced by vitamin K absence or antagonists II (PIVKA-II) every month, and by abdominal ultrasound, dynamic CT scan or dynamic MRI every 3 months. Recurrent HCC was diagnosed using the imaging modalities described earlier as the appearance of another lesion different from the primary one. 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 2008 if HCC did not recur. We defined the local tumor progression at the initial HCC site as censored.

### Statistical analysis

Baseline characteristics were compared using the Student's *t*-test for continuous variables or  $\chi^2$ -test for categorical variables. Recurrence-free survival was estimated using the Kaplan–Meier method, and differences between curves were examined by log–rank test. There were 13 possible predictors for recurrence of HCC after the initial curative treatment: sex, age, body mass index (BMI), Child–Pugh classification, serum albumin level, total bilirubin level, alanine aminotransferase (ALT) activity, platelet count, prothrombin time, HOMA-IR (defined as fasting plasma glucose [mg/dL]  $\times$  fasting immunoreactive insulin [ $\mu$ U/mL] / 405), hemoglobin A<sub>1c</sub> (HbA<sub>1c</sub>), and serum tumor markers (AFP and PIVKA-II). The parameters, that proved to be significant by log–rank test, were then subjected to the univariate and multivariate analyses using the Cox proportional hazards model. Statistical significance was declared if the *P*-value was 0.05 or less. In addition, we employed the quantitative insulin sensitivity check index (QUICKI), which directly correlates with the glucose clamp method,<sup>17</sup> to supplement the evaluation of insulin resistance by HOMA-IR: QUICKI =  $1 / (\log [\text{immunoreactive insulin}] + \log [\text{fasting plasma glucose}])$ .

## RESULTS

### Patients' baseline characteristics and laboratory data

THE BASELINE CHARACTERISTICS and laboratory data of 24 patients (15 men and nine women, median age 73 years) are shown in Table 1. Twenty

**Table 1** Baseline demographic and clinical characteristics

		Normal range
Sex (male/female)	15/9	
Age (years)	73 (61–82)	
BMI	22.3 (19.5–33.5)	
Child–Pugh classification (A/B/C)	20/4/0	
Follow-up period (days)	365 (60–770)	
ALB (g/dL)	3.75 (2.4–4.4)	3.9–4.9
ALT (IU/L)	48.5 (21–98)	7–40
T-Bil (mg/dL)	0.96 (0.6–2.1)	0.2–1.2
PLT ( $\times 10^4/\mu\text{L}$ )	8.85 (4.1–21)	14.1–32.7
PT (%)	74 (56–118)	70–120
FPG (mg/dL)	107 (75–155)	70–110
FIRI ( $\mu\text{g/dL}$ )	10.8 (2.78–32.2)	2–10
HOMA-IR	2.96 (0.76–7.39)	<1.6
HbA1c (%)	5.2 (3.7–7.2)	<5.6
AFP (ng/dL)	26.7 (2.2–203)	<20
PIVKA-II (mAU/mL)	24 (9–127)	<40

Values are median (range).

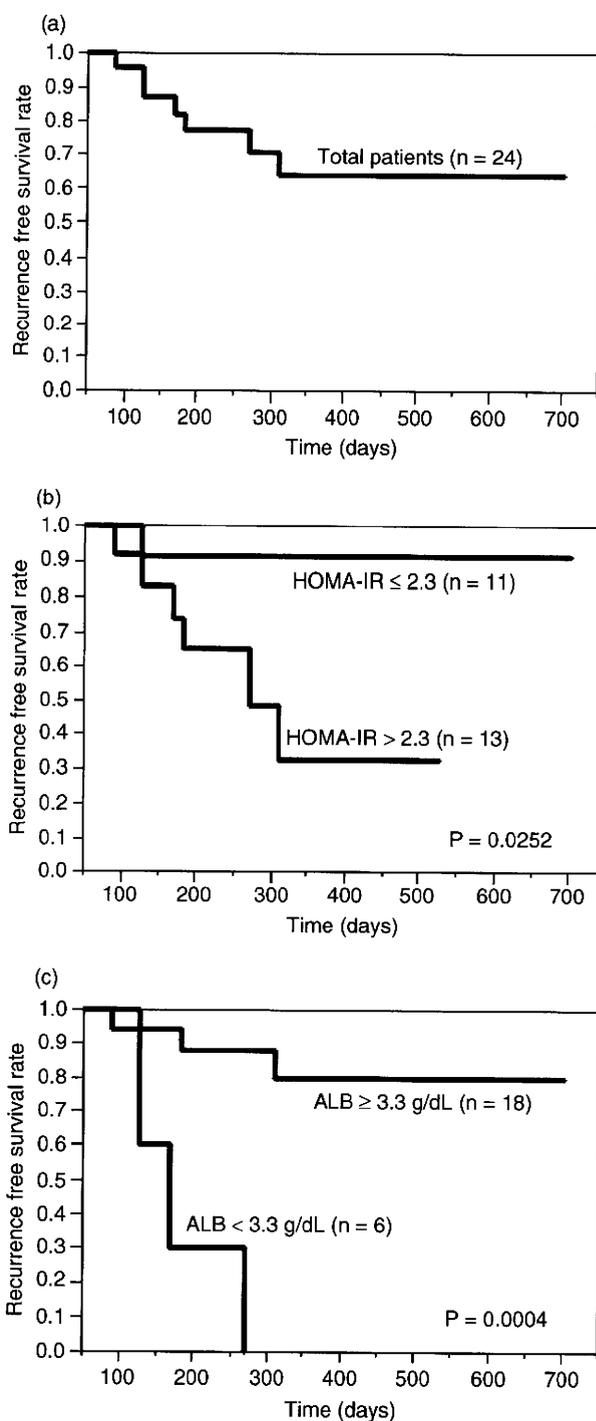
AFP,  $\alpha$ -fetoprotein; ALB, albumin; ALT, alanine aminotransferase; BMI, body mass index; FIRI, fasting immunoreactive insulin; FPG, fasting plasma glucose; HOMA-IR, Homeostatic Model Assessment of Insulin Resistance; PIVKA-II, protein induced by vitamin K absence or antagonists II; PLT, platelets; PT, prothrombin time; T-Bil, total bilirubin.

patients were classified into Child–Pugh class A, four patients into class B and none into class C. The median follow-up period was 365 days (range 60–770 days), and no patient died during the study.

### Possible risk factors for recurrence of HCC

No local tumor progression was diagnosed in this study period. Seven patients experienced the defined recurrence in the liver, but no one showed distant metastasis. One-year recurrence-free survival in total patients was 64%; Figure 1(a,b) shows Kaplan–Meier curves for recurrence-free survival according to HOMA-IR level ( $\leq 2.3$  and  $> 2.3$ ), which produced significant difference ( $P = 0.0252$ ). Serum albumin level ( $\geq 3.3$  and  $< 3.3$  g/dL;  $P = 0.0004$ ) was also a significant variable (Fig. 1c).

The Cox proportional hazards model was used to analyze risk factors for recurrence of stage I HCC after the curative RFA, using the 13 variables described earlier (Table 2). HOMA-IR level (odds ratio [OR] = 1.66, 95% CI = 1.01–2.72,  $P = 0.0420$ ), and serum albumin level (OR = 0.08, 95% CI = 0.01–0.45,  $P = 0.0036$ ) were identified as significant risk factors by univariate analysis. Multivariate analysis identified albumin (OR = 0.01,



**Figure 1** Kaplan–Meier curves for recurrence-free survival in (a) total patients and in subgroups divided according to (b) Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) level or (c) serum albumin level. ALB, albumin.

**Table 2** Univariate analyses of possible risk factors for recurrence of hepatocellular carcinoma by Cox proportional hazards model

	OR	95% CI		P-value
		Lower	Upper	
Men (vs women)	1.20	0.25	8.41	0.8242
Age (years)	1.06	0.93	1.23	0.3451
BMI	0.90	0.59	1.22	0.6036
Child B (vs A)	4.81	0.60	31.3	0.1253
ALB (g/dL)	0.08	0.01	0.45	0.0036
T-Bil (mg/dL)	2.75	0.27	19.7	0.3603
ALT (IU/L)	0.99	0.95	1.02	0.6923
PLT ( $\times 10^4/\mu\text{L}$ )	0.86	0.65	1.05	0.1770
PT (%)	0.95	0.87	1.01	0.1617
HOMA-IR	1.66	1.01	2.72	0.0420
HbA1c (%)	0.69	0.27	1.53	0.3850
AFP (ng/dL)	1.00	0.99	1.02	0.1242
PIVKA-II (mAU/mL)	1.00	0.96	1.03	0.6172

OR is shown with a unit increase in continuous variables. AFP,  $\alpha$ -fetoprotein; ALB, albumin; ALT, alanine aminotransferase; BMI, body mass index; CI, confidence interval; FIRI, fasting immunoreactive insulin; FPG, fasting plasma glucose; HOMA-IR, Homeostatic Model Assessment of Insulin Resistance; OR, odds ratio; PIVKA-II, protein induced by vitamin K absence or antagonists II; PLT, platelets; PT, prothrombin time; T-Bil, total bilirubin.

95% CI = 0.0002–0.15,  $P = 0.0001$ ) and HOMA-IR level (OR = 3.85, 95% CI = 1.57–14.2,  $P = 0.0015$ ) as significant independent risk factors for recurrence.

Table 3 shows the patients' baseline characteristics and laboratory data divided according to HOMA-IR level ( $\leq 2.3$  and  $> 2.3$ ). No significant differences were noted between the two subgroups except fasting plasma glucose and fasting immunoreactive insulin. Two patients in the HOMA-IR 2.3 or less subgroup took oral hypoglycemic drugs, sulfonylurea derivatives and voglibose. Three patients in the HOMA-IR more than 2.3 subgroup took oral hypoglycemic drugs; two took sulfonylurea derivatives and one took pioglitazone. No patient received insulin treatment.

We supplementally analyzed the data by excluding the patients under treatment with these oral hypoglycemics and also the patients with fasting plasma glucose above 140 mg/dL, in order to avoid possible unreliability in HOMA-IR evaluation. In nine patients, each remaining in HOMA-IR of 2.3 or less and HOMA-IR of more than 2.3, serum albumin (OR = 0.02, 95% CI = 0.0002–0.40,  $P = 0.0060$ ) and HOMA-IR (OR = 3.49, 95% CI = 1.45–13.8,  $P = 0.0033$ ) were still significant.

In a similar manner, evaluation of insulin sensitivity by QUICKI gave the results that lower QUICKI ( $\leq 0.33$ ,

**Table 3** Baseline demographic and clinical characteristics of patients classified according to HOMA-IR level

	HOMA-IR $\leq 2.3$ ( $n = 11$ )	HOMA-IR $> 2.3$ ( $n = 13$ )	P-value
Sex (male/female)	7/4	8/5	0.9157
Age (years)	70 (61–82)	74 (63–80)	0.1846
BMI	23.55 (19.5–33.5)	22.1 (19.5–25.1)	0.1219
Follow-up period (days)	393 (155–701)	337 (60–770)	0.2785
Child–Pugh classification (A/B)	10/1	10/3	0.3483
ALB (g/dL)	3.9 (2.4–4.4)	3.4 (2.7–4.4)	0.3304
ALT (IU/L)	40 (21–98)	53 (25–80)	0.6103
T-Bil (mg/dL)	0.8 (0.7–2.1)	1.0 (0.6–1.7)	0.6655
PLT ( $\times 10^4/\mu\text{L}$ )	8.5 (4.1–21)	8.9 (4.9–13.9)	0.5766
PT (%)	72 (56–95.5)	74 (58–118)	0.9953
FPG (mg/dL)	90 (75–119)	109 (86–155)	0.0151
FIRI ( $\mu\text{g}/\text{dL}$ )	7.98 (2.78–10.8)	14.1 (7.86–32.2)	0.0005
HOMA-IR	1.79 (0.76–2.27)	3.76 (2.91–7.39)	$< 0.0001$
HbA1c (%)	5.05 (3.7–7.2)	5.3 (4.1–6.8)	0.6848
AFP (ng/dL)	23.2 (2.2–153.2)	28 (8–203)	0.7339
PIVKA-II (mAU/mL)	21 (9–127)	28 (9–67)	0.8071
Presence of oral hypoglycemic drugs (yes/no)	2/9	3/10	0.7678
Presence of insulin treatment (yes/no)	0/11	0/13	1.0000

Values are median (range).

AFP,  $\alpha$ -fetoprotein; ALB, albumin; ALT, alanine aminotransferase; BMI, body mass index; FIRI, fasting immunoreactive insulin; FPG, fasting plasma glucose; HOMA-IR, Homeostatic Model Assessment of Insulin Resistance; PIVKA-II, protein induced by vitamin K absence or antagonists II; PLT, platelets; PT, prothrombin time; T-Bil, total bilirubin.

i.e. impaired insulin sensitivity) was associated significantly with the increased risk of HCC recurrence (OR = 7.97, 95% CI = 1.32–152,  $P = 0.0213$ ).

## DISCUSSION

SEVERAL EPIDEMIOLOGICAL STUDIES have revealed the association of DM with cancer incidence and cancer mortality for various organs such as the liver, biliary tract, pancreas, endometrium, kidney, colon, bladder and breast.<sup>7,18–21</sup> The mechanism by which insulin acts as a carcinogenic factor is currently a focus of interests. First, insulin functions as a growth factor by phosphorylating insulin receptor substrate 1 and activating the downstream mitogen-activated protein kinase cascade, which affects cellular proliferation.<sup>22,23</sup> Second, hyperinsulinemia increases peripheral lipolysis and hepatic accumulation of free fatty acids, and the excess  $\beta$ -oxidation in mitochondria and microsome leads to the production of reactive oxygen species<sup>24,25</sup> that play a significant role in carcinogenesis.<sup>26,27</sup> Adipocyte-secreted cytokines (adipokines) such as tumor necrosis factor- $\alpha$  and interleukin-6 also play a significant role in both insulin resistance and carcinogenesis.<sup>28,29</sup> Thus, these factors could cooperatively induce the insulin resistance and carcinogenesis.

We demonstrated in the present study that a higher HOMA-IR level increases the risk of early recurrence after initial curative RFA of stage I HCC in HCV-positive patients. This finding basically agrees with previous studies<sup>7,8,18</sup> that suggested an association between insulin resistance and carcinogenesis as described above, but HbA<sub>1c</sub> level did not predict recurrence (Table 2). This might be explained by the clinical relevance that HbA<sub>1c</sub> level in patients with liver cirrhosis is often underestimated because of anemia. Therefore, the results of the present study suggest that the role of hyperinsulinemia is more important than that of hyperglycemia as reflected by HbA<sub>1c</sub> in the recurrence of HCC. We therefore should pay attention to levels not only of glucose and HbA<sub>1c</sub> but also of insulin when we follow patients who are at risk for HCC.

Interventional modalities to improve insulin-resistance could be a key to prevent the primary or recurrent HCC in patients complicated with such metabolic disorders. For instance, metformin and thiazolidine derivatives could be potential candidates for this purpose.<sup>30,31</sup> Oral branched-chain amino acid (BCAA) granules might be a candidate for preventing HCC recurrence in DM cases because, in addition to improv-

ing hypoalbuminemia,<sup>32,33</sup> this agent improves insulin resistance without stimulating insulin secretion.<sup>34</sup> Improvements of insulin resistance and glucose tolerance by BCAA have been reported in clinical trials.<sup>35,36</sup> Furthermore, Muto *et al.*<sup>37</sup> described that oral supplementation with BCAA granules inhibited liver carcinogenesis in HCV-positive liver cirrhosis with DM and obesity. Such effect of BCAA is also supported in experimental models.<sup>38,39</sup> These reports,<sup>32–39</sup> together with our present findings (Table 2 and Fig. 1b), suggest that insulin resistance is a significant risk factor for early recurrence of HCC and thus might be a critical target to prevent the recurrence and development of second primary HCC.

A limitation of this study is that the therapeutic effect of the primary HCC was judged as curative by imaging diagnosis but not by surgical pathology. Although the recurrent HCC developed apart from the primary tumor, we could not totally differentiate the recurrent lesion and a second primary HCC. A higher recurrence rate in this study (Fig. 1a) might be explained by this fact. Advanced medical imagings such as positron emission tomography would help solving such limitations in future study of this kind. Furthermore, the basic question, if *de novo*, namely, the first or second primary liver carcinogenesis is regulated by insulin resistance, should be addressed by recruiting HCC-free cirrhotics. However, such study requires a larger sample size and a longer observation period. Instead, we focused on the recurrent HCC, including possible second primary tumors, which develop at a 2–3-fold higher incidence than the first primary one.

Other study limitations are the short observation period, the small number of recruited patients and also the small number of detected events. Such a sample size essentially raises the possibility of  $\beta$ -error, including the absence of the statistical power of AFP and PIVKA-II for predicting the recurrence (Table 2). Previous reports agree that these tumor markers are risk factors of HCC recurrence,<sup>2,6</sup> although some criticisms remain.<sup>4,5</sup> In addition, we should state that the small number of events particularly restricts the reliability of multivariate analysis, while the calculation itself was possible in our study.

In conclusion, we presented for the first time that insulin resistance is significantly associated with the early recurrence of stage I HCC after curative RFA in HCV-positive patients. Increased HOMA-IR, which sensitively reflects insulin resistance, might be a useful biomarker for prediction of high-risk patients who cause early recurrence of HCC.