10. Porchet N, Pigny P, Buisine MP, Debailleul V, Degand P, Laine A and Aubert JP: Human mucin genes: genomic organization and expression of MUC4, MUC5AC and MUC5B. Biochem Soc Trans 23: 800-805, 1995.
11. Shankar V, Pichan P, Eddy RL Jr, *et al*: Chromosomal localization of a human mucin gene (MUC8) and cloning of the cDNA corresponding to the carboxy terminus. Am J Respir Cell Mol Biol 16: 232-241, 1997.
12. Toribara NW, Roberton AM, Ho SB, *et al*: Human gastric mucin. Identification of a unique species by expression cloning. J Biol Chem 268: 5879-5885, 1993.
13. Williams SJ, Wreschner DH, Tran M, Eyre HJ, Sutherland GR and McGuckin MA: Muc13, a novel human cell surface mucin expressed by epithelial and hemopoietic cells. J Biol Chem 276: 18327-18336, 2001.
14. Yin BW and Lloyd KO: Molecular cloning of the CA125 ovarian cancer antigen: identification as a new mucin, MUC16. J Biol Chem 276: 27371-27375, 2001.
15. Bafna S, Singh AP, Moniaux N, Eudy JD, Meza JL and Batra SK: MUC4, a multifunctional transmembrane glycoprotein, induces oncogenic transformation of NIH3T3 mouse fibroblast cells. Cancer Res 68: 9231-9238, 2008.
16. Velcich A, Yang W, Heyer J, *et al*: Colorectal cancer in mice genetically deficient in the mucin Muc2. Science 295: 1726-1729, 2002.
17. Balague C, Gambus C, Carrato N, Porchet N, Aubert JP, Kim YS and Real FX: Altered expression of MUC2, MUC4, and MUC5 mucin genes in pancreas tissues and cancer cell lines. Gastroenterology 106: 1054-1061, 1994.
18. Voynow JA, Young LR, Wang Y, Horger T, Rose MC and Fischer BM: Neutrophil elastase increases MUC5AC mRNA and protein expression in respiratory epithelial cells. Am J Physiol 276: L835-L843, 1999.
19. Hovenberg HW, Davies JR and Carlstedt I: Different mucins are produced by the surface epithelium and the submucosa in human trachea: identification of MUC5AC as a major mucin from the goblet cells. Biochem J 318: 319-324, 1996.
20. Hovenberg HW, Davies JR, Herrmann A, Lindén CJ and Carlstedt I: MUC5AC, but not MUC2, is a prominent mucin in respiratory secretions. Glycoconj J 13: 839-847, 1996.
21. Sheehan JK, Brazeau C, Kutay S, Pigeon H, Kirkham S, Howard M and Thornton DJ: Physical characterization of the MUC5AC mucin: a highly oligomeric glycoprotein whether isolated from cell culture or *in vivo* from respiratory mucous secretions. Biochem J 347: 37-44, 2000.
22. Janek P, Briand P and Hartman NR: The effect of estrone-progesterone treatment on cell proliferation kinetics of hormone-dependent GR mouse mammary tumors. Cancer Res 35: 3698-3704, 1975.
23. Tsutsumida H, Swanson BJ, Singh PK, Caffrey TC, Kitajima S, Goto M, Yonezawa S and Hollingsworth MA: RNA interference suppression of MUC1 reduces the growth rate and metastatic phenotype of human pancreatic cancer cells. Clin Cancer Res 12: 2976-2987, 2006.
24. Chaturvedi P, Singh AP, Moniaux N, Senapati S, Chakraborty S, Meza JL and Batra SK: MUC4 mucin potentiates pancreatic tumor cell proliferation, survival, and invasive properties and interferes with its interaction to extracellular matrix proteins. Mol Cancer Res 5: 309-320, 2007.
25. Truant S, Bruyneel E, Gouyer V, De Wever O, Pruvot FR, Mareel M and Huet G: Requirement of both mucins and proteoglycans in cell-cell dissociation and invasiveness of colon carcinoma HT-29 cells. Int J Cancer 104: 683-694, 2003.
26. Ligtenberg MJL, Buijs F, Vos HL and Hilkens J: Suppression of cellular aggregation by high levels of episialin. Cancer Res 52: 2318-2324, 1992.
27. Duan R, Remeijer L, van Dun JM, Osterhaus AD and Verjans GM: Granulocyte macrophage colony-stimulating factor expression in human herpetic stromal keratitis: implications for the role of neutrophils in HSK. Inv Ophth Vis Sci 48: 277-284, 2007.
28. Tamamori Y, Sawada T, Nishihara T, *et al*: Granulocyte-colony stimulating factor enhances chimeric antibody K-MAC5 dependent cytotoxicity against pancreatic cancer mediated by polymorphonuclear neutrophils. Int J Oncol 21: 649-654, 2002.
29. Lieschke GJ and Burgess AW: Drug therapy: granulocyte colony-stimulating factor and granulocyte-macrophage colony-stimulating factor (1&2). N Engl J Med 327: 28-35, 99-106, 1992.
30. Stockmeyer B, Valerius T, Repp R, *et al*: Preclinical studies with FcγR bispecific antibodies and granulocyte colony-stimulating factor-primed neutrophils as effector cells against HER-2/neu overexpressing breast cancer. Cancer Res 57: 696-701, 1997.
31. Hinoda Y, Ikematsu Y, Horinochi M, *et al*: Increased expression of MUC1 in advanced pancreatic cancer. J Gastroenterol 38: 1162-1166, 2003.
32. Gimmi CD, Morrison BW, Mainprice BA, *et al*: Brest cancer associated antigen, DF3/MUC1, induces apoptosis of activated human T cells. Nat Med 2: 1367-1370, 1996.
33. Ho JJL: Mucins in the diagnosis and therapy of pancreatic cancer. Curr Pharm Des 6: 1881-1896, 2000.
34. Inaba T, Sano H, Kawahito Y, *et al*: Induction of cyclo-oxygenase-2 in monocyte/macrophage by mucins secreted from colon cancer cells. Proc Natl Acad Sci USA 100: 2736-2741, 2003.
35. Sugihara I, Yoshida M, Shigenobu T, *et al*: Different progression of tumor xenografts between mucin-producing and mucin-non-producing mammary adenocarcinoma-bearing mice. Cancer Res 66: 6175-6182, 2006.

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**Original**

# Enhanced Urinary Bladder, Liver and Colon Carcinogenesis in Zucker Diabetic Fatty Rats in a Multiorgan Carcinogenesis Bioassay: Evidence for Mechanisms Involving Activation of PI3K Signaling and Impairment of p53 on Urinary Bladder Carcinogenesis

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**Abstract**: In the present study, modifying effects of diabetes on carcinogenesis induced in type 2 diabetes mellitus model Zucker diabetic fatty (ZDF) rats were investigated using a multiorgan carcinogenesis bioassay. Our results demonstrated enhancement of urinary bladder, colon and liver carcinogenesis in ZDF rats treated with five types of carcinogens (DMBDD). Elevated insulin and leptin and decreased adiponectin levels in the serum may be responsible for the high susceptibility of type 2 diabetes mellitus model rats to carcinogenesis in these organs. Possible mechanisms of increased susceptibility of diabetic rats to bladder carcinogenesis could be activation of the PI3K pathway and suppression of p53 in the urothelium in consequence of the above serum protein alterations. (DOI: 10.1293/tox.24.25; J Toxicol Pathol 2011; **24**: 25–36)

**Key words**: type 2 diabetes mellitus, bladder carcinogenesis, PI3K, p53

## Introduction

Type 2 diabetes mellitus (T2DM) has been associated with a number of complications, such as cardiovascular disease, diabetic nephropathy and infection[1]. Recently, with the markedly improved survival of T2DM patients, attributed to the successful treatment of cardiovascular disease and infection control, the relationship between T2DM and cancer has been attracting attention. In epidemiological studies, T2DM was reported to be associated with increased risks of colon, pancreas, mammary, liver and urinary bladder cancers; however, the underlying mechanisms of increased cancer risks in T2DM patients remain unclear[2,3].

The influence of diabetes on carcinogenesis induced by chemical carcinogens has been investigated in the colon, stomach and mammary gland in experimental animals[4–6]. The number of colon tumors initiated by 1,2-dimethylhydrazine dihydrochloride (DMH) was increased in Otsuka Long-Evans Tokushima Fatty (OLETF) rats spontaneously developing T2DM compared with LETO rats, a non-diabetic strain[4]. Insulin resistance and hyperinsulinemia were suggested as direct risk factors for colon cancer[7]. Furthermore, db/db diabetic mice were reported to be highly susceptible to stomach carcinogenesis induced by *N*-methyl-*N*-nitrosourea (MNU), possibly in association with hyperinsulinemia and hyperleptinemia[5]. On the other hand, decreased susceptibility to MNU-induced mammary carcinogenesis was reported in streptozotocin-induced diabetic rats, possibly due to significantly lowered plasma levels of insulin and insulin-like growth factor 1 (IGF-1) during the promotion phase of carcinogenesis[6]. Based on these findings, metabolic abnormalities accompanying diabetes, such as hyperinsulinemia, hyperleptinemia and a high plasma level of IGF-1, have been proposed to play a role in cancer development in DM patients.

Zucker Diabetic Fatty (ZDF) rats closely mimic human adult onset T2DM and its related complications due to the inherited homozygous leptin receptor mutation, which leads to obesity and insulin resistance[8]. T2DM does not develop


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in lean littermate (Lean) animals[8]. ZDF animals show insulin resistance from the time of weaning but maintain normoglycemia until 8–10 weeks of age because of the compensatory hypersecretion of insulin[9]. ZDF rats develop overt diabetes and have defects in insulin secretion from around 8–10 weeks of age[10].

The purpose of the present study was to investigate the modifying effects of T2DM on carcinogenesis in diabetic ZDF rat using a multiorgan carcinogenesis bioassay. The multiorgan carcinogenesis bioassay uses treatment with five types of genotoxic carcinogens, namely *N*-diethylnitrosamine (DEN), MNU, *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine (BBN), diisopropanolnitrosamine (DHPN) and DMH, with target organs including the liver, kidneys, urinary bladder, stomach, small intestine, colon, lungs and thyroid[11,12]. At the end of the experiment, histopathological analysis was performed to investigate cancer development. We also investigated the serum levels of several biologically active compounds, including insulin, leptin, adiponectin and IGF-1. Furthermore, to elucidate possible mechanisms underlying the modifying effects of T2DM, we analyzed alterations of the gene expression in the bladder urothelium in a 4-week BBN bladder carcinogenicity study.

## Materials and methods

### *Animals*

Five-week-old ZDF and control Lean rats were purchased from Charles River Laboratories Japan, Inc. (Kanagawa, Japan) and used after a 1-week acclimation period. They were housed in plastic cages (one rat/cage for ZDF rats, two rats/cage for Lean rats) in an environmentally-controlled room maintained at a temperature of 22 ± 2°C and relative humidity of 44 ± 5%, with a 12-h light/dark cycle and free access to water and food (MF pellet diet; Oriental Yeast Co., Ltd., Tokyo, Japan). Body weight and food intake were measured weekly during the experimental period. Water intake was measured three times a week in the first 4 weeks and once a week thereafter.

### *Chemicals*

DEN, DMH and BBN were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). MNU was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), and DHPN was purchased from Nacalai Tesque Inc. (Kyoto, Japan).

### *Experimental protocol*

Experimental protocols were approved by the Institution Animal Care and Use Committee of Osaka City University Medical School. The experimental protocol of the rat multiorgan carcinogenesis bioassay used in experiment 1 is shown in Fig. 1[11,12]. Twenty-six male ZDF rats at 6 weeks of age were divided randomly into two groups. Twenty ZDF rats were treated with five carcinogens, DEN, MNU, BBN, DMH and DHPN (DMBDD), as follows: a single i.p. injection of DEN (100 mg/kg b.w.) at the beginning of the experiment, followed by four i.p. injections of MNU (20 mg/kg b.w.) from week 1 to 2, and four s.c. injections of DMH (40 mg/kg b.w.) from week 3 to 4. Also, 0.05% BBN in drinking water was administered to rats for two weeks from commencement of the experiment, and then DHPN in drinking water was administered to the rats for the following 2 weeks. Six control ZDF rats were administered tap water and received vehicle injections (0.9% saline) in the same manner. Twenty-seven male Lean rats at 6 weeks of age were divided randomly into two groups and treated with DMBDD (20 rats) and vehicle (7 rats), respectively, as described above. DHPN was administered to Lean rats at a concentration of 0.1% in drinking water. As ZDF rats were drinking more water than Lean rats from week 3 to 4, the concentrations of DHPN administered to the ZDF rats were diluted to give the same amount of DHPN per b.w. as the Lean rats based on their body weight and water intake. After the initiation treatments, all rats were maintained without any treatment until the end of the study. Blood glucose was examined weekly from week 3 to 7 and once every two weeks from week 8 to 30 via the tail vein using a blood glucose test meter (Glutest Ace R; Sanwa Kagaku Kenkyusho Co., Ltd., Nagoya, Japan). At the end of week 30, all surviving animals were euthanized under deep anesthesia, target organs were taken out and the liver, kidney and spleen weights were measured. All target organs were fixed in 10% phosphate-buffered formalin and embedded in paraffin for histopathological examination. Blood serum was collected for biochemical analyses. Part of the liver tissues was snap frozen in liquid nitrogen and stored at −80°C.

In experiment 2, 12, 6-week-old male ZDF or Lean rats were divided randomly into two groups each. BBN in drinking water was administered for 4 weeks from commencement of the experiment to ZDF (6) or Lean (6) rats. The remaining 6 ZDF and 6 Lean rats were administered tap water. Given the differences in water intake and body weight between the ZDF and Leans rats, water intake and body weights were measured three times a week, and the BBN concentration was adjusted to give the same amount of BBN per b.w. to both strains of rats based on the water intake per body weight per day from commencement of the experiment. Briefly, 0.05% BBN was administered to the strain of rats drinking less water (g/kg, b.w.), and the concentration of BBN was diluted and administered to the strain of rats drinking more water. Blood glucose was examined weekly from week 1 to 4. At week 4, all rats were euthanized under deep anesthesia, and blood serum was collected for biochemical analyses. The bladder mucosa was harvested using the method described previously[13]. Part of the liver tissues was snap frozen in liquid nitrogen and stored at −80°C.

### *Blood biochemistry analysis*

Blood biochemistry was analyzed at the end of the experiments (week 30 in experiment 1 and week 4 in experiment 2) by Mitsubishi Chemical Medience Corporation (Tokyo, Japan). The serum levels of insulin, leptin, adiponectin, IGF-1, tumor necrosis factor α (TNFα) and inter-

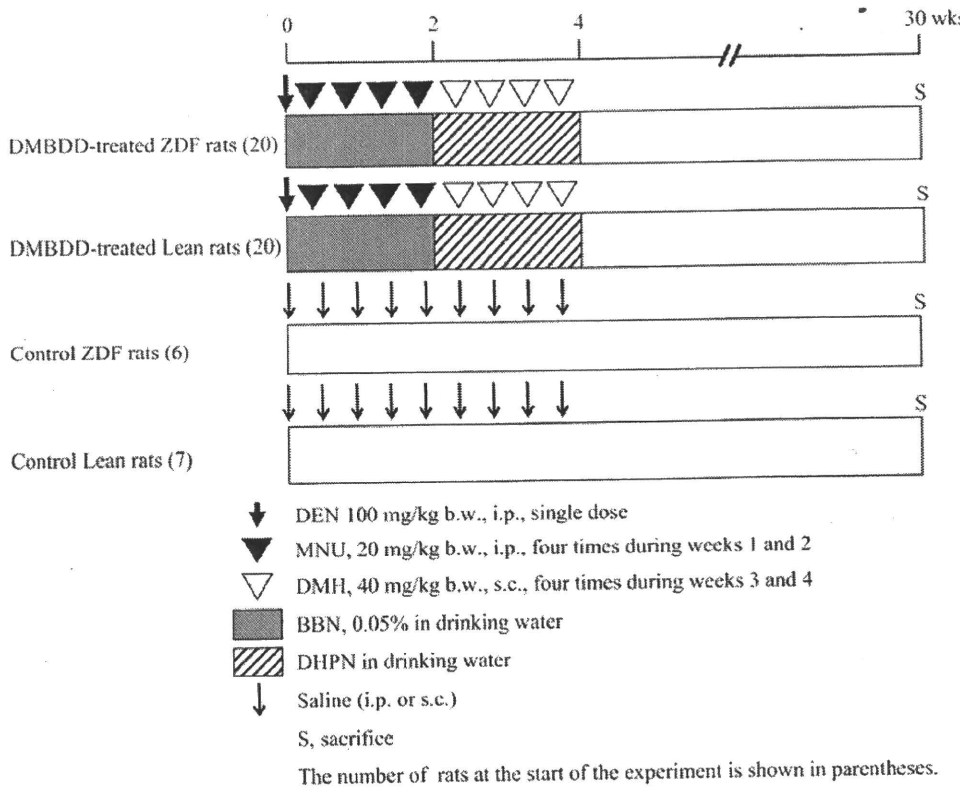


**Fig. 1.** Experimental protocol of experiment 1 (DMBDD model).

leukin-6 (IL-6) of all surviving rats were measured using rat ELISA kits (insulin, Shibayagi Co., Ltd., Gunma, Japan; leptin, Morinaga Institute of Biological Science, Inc., Kanagawa, Japan; adiponectin, Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan; IGF-1, TNFα and IL-6, R&D Systems, Inc., Minneapolis, MN, USA) according to the manufacturers' instructions.

*RNA preparation and real-time quantitative PCR*

RNA from the bladder mucosa and liver was isolated using TRIZOL Reagent (Life Technologies Japan Ltd., Tokyo, Japan) according to the manufacturer's instructions. Synthesis of cDNA was performed with 600 ng RNA using an Advantage RT-for-PCR kit (Takara Bio, Inc., Shiga, Japan). Real-time quantitative PCRs for phosphatidylinositol 3-kinase (PI3K), p53, PCNA and β-actin as an internal control for the bladder mucosa and IGF-1 and rsp18 as an internal control for the liver were performed using an Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, Inc., Tokyo, Japan) as described previously[13,14]. Briefly, 20 μl containing 1 μl of the respective TaqMan Gene Expression Assays (Applied Biosystems, Inc., Tokyo, Japan), 10 μl TaqMan Fast Universal PCR Master Mix (Applied Biosystems, Inc., Tokyo, Japan) and 5 μl diluted cDNA were applied to a Fast 96-well Reaction Plate.

Serially diluted standard cDNA was included in each TaqMan PCR reaction to create standard curves. The amounts of gene products in the test samples were estimated relative to the respective standard curves. Values for target genes were normalized to those for β-actin or rsp18.

*Statistical analysis*

All mean values are reported as means ± SD. Statistical analyses were performed using the StatLight program (Yukms Co., Ltd., Tokyo, Japan). Homogeneity of variance analysis was performed by the F test. Differences in mean values between the control and carcinogen-treated groups were evaluated by Student's *t*-test when the variance was homogeneous and Welch's *t*-test when the variance was heterogeneous. Incidence was assessed by Fisher's exact probability test. *P* values less than 0.05 were considered significant.

## Results

*General observations*

Experiment 1: Five DMBDD-treated ZDF rats were found dead or moribund at weeks 17, 21, 22, 27 and 29. Furthermore, two control ZDF rats were found dead or moribund without any discernible cause at weeks 21 and 26. All DMBDD-treated and control Lean rats were alive at the end of the study. As bladder, small intestine and liver tumors

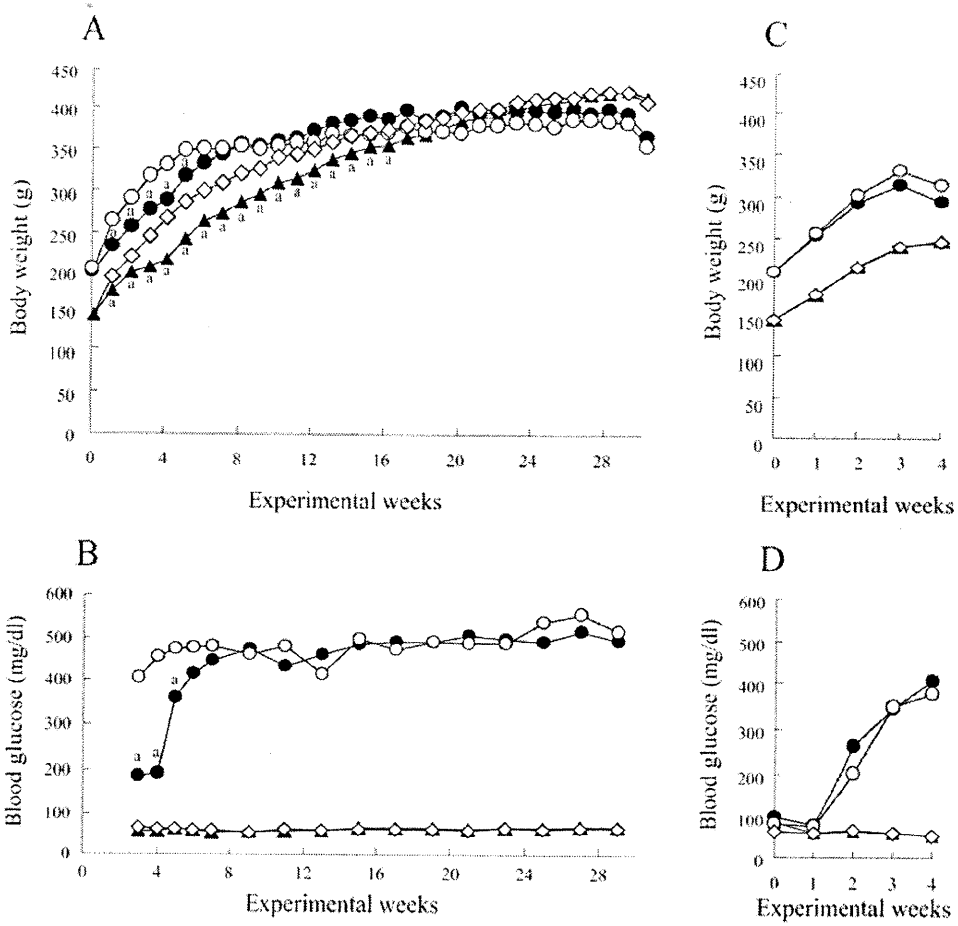


**Fig. 2.** Time course of body weight and blood glucose level in experiment 1 (A, B) and experiment 2 (C, D).
[a]: $P<0.05$ vs. control rats of the same genotype.

were found in the DMBDD-treated ZDF rat that died at week 17, all rats were included in the effective animals for histopathological analysis.

Body weight curves, final body weight and absolute and relative organ weights of the rats in experiment 1 are shown in Fig. 2A and Table 1. The body weights of both the DMBDD-treated ZDF and Lean rats were significantly decreased during administration of DMBDD compared with the control rats of the same genotype; however, the final body weights of both the ZDF and Lean rats administered DMBDD were not significantly changed as compared with the control rats of the same genotype. The body weights of the ZDF rats were significantly higher during the DMBDD treatment period but were significantly lower than those of the Lean rats at the end of the study irrespective of whether or not they received carcinogen treatment.

During BBN treatment (weeks 1 and 2), the total BBN intake of the ZDF rats (0.54 ± 0.07 mg/kg b.w.) was significantly lower than that of the Lean rats (0.63 ± 0.04 mg/kg b.w.). During DHPN treatment (weeks 3 and 4), almost the same total amount of DHPN was given to both strain rats (1.3 ± 0.4 mg/kg b.w. for the ZDF rats and 1.3 ± 0.1 mg/kg b.w. for the Lean rats) by adjusting the concentration of DHPN based on the water intake and body weight, as described in the Methods.

DMBDD administration inhibited food intake of both the ZDF and Lean rats compared with the control rats of the same genotype. Food intake was significantly increased in the DMBDD-treated and non-treated ZDF rats when compared with the Lean rats receiving the same treatment throughout the experiment (data not shown).

The absolute liver and spleen weights and relative spleen weight of the DMBDD-treated ZDF rats were significantly higher than those of the control ZDF rats. Furthermore, the absolute and relative spleen weights in the DMBDD-treated Lean group were significantly elevated compared with those of the Lean control animals.

Experiment 2: All rats survived to the end of the study.

**Table 1.** Final Body and Organ Weights (Experiment 1)

| | DMBDD | | Control | |
|---|---|---|---|---|
| | ZDF | Lean | ZDF | Lean |
| Initial no. of rats | 20 | 20 | 6 | 7 |
| Final no. of rats | 15 | 20 | 4 | 7 |
| Survival rate (%) | 75 | 100 | 67 | 100 |
| Final body weight (g) | 367 ± 19 | 416 ± 24 | 355 ± 41 | 411 ± 32 |
| Absolute (g) | | | | |
| Liver | 18.0 ± 2.4[a] | 10.6 ± 0.9 | 15.0 ± 3.8 | 10.9 ± 0.9 |
| Kidneys | 4.4 ± 2.7 | 2.3 ± 0.1 | 3.6 ± 0.3 | 2.2 ± 0.2 |
| Spleen | 0.9 ± 0.5[a] | 0.6 ± 0.1[a] | 0.4 ± 0.2 | 0.5 ± 0.0 |
| Relative (g/100g bw) | | | | |
| Liver | 4.9 ± 0.6 | 2.5 ± 0.1[a] | 4.4 ± 0.9 | 2.7 ± 0.1 |
| Kidneys | 1.2 ± 0.7 | 0.5 ± 0.0 | 1.1 ± 0.1 | 0.5 ± 0.0 |
| Spleen | 0.2 ± 0.2[a] | 0.2 ± 0.0[a] | 0.1 ± 0.0 | 0.1 ± 0.0 |

[a] $P<0.05$ vs. control rats of the same genotype.

**Table 2.** Blood Biochemistry

| Experiment 1 | DMBDD | | Control | |
|---|---|---|---|---|
| week 30 | ZDF | Lean | ZDF | Lean |
| HbA1c (%) | 8.3 ± 0.7*,[a] | 3.3 ± 0.1 | 9.6 ± 0.5* | 3.3 ± 0.1 |
| Glucose (mg/dl) | 479 ± 45* | 137 ± 22 | 512 ± 51* | 129 ± 8 |
| T-Cho (mg/dl) | 241 ± 69*,[a] | 92 ± 19 | 404 ± 98* | 92 ± 10 |
| TG (mg/dl) | 740 ± 353* | 51 ± 14 | 1053 ± 404* | 61 ± 10 |
| AST (IU/l) | 390 ± 222* | 159 ± 38 | 618 ± 208* | 148 ± 35 |
| ALT (IU/l) | 428 ± 265*,[a] | 61 ± 51[a] | 783 ± 353* | 32 ± 3 |
| γ-GTP (IU/l) | 27 ± 15*,[a] | 2 ± 1[a] | 72 ± 28* | 1 ± 0 |
| Experiment 2 | BBN | | Control | |
| week 4 | ZDF | Lean | ZDF | Lean |
| HbA1c (%) | 5.8 ± 0.6* | 3.2 ± 0.0 | 5.6 ± 0.6* | 3.2 ± 0.0 |
| Glucose (mg/dl) | 311 ± 62* | 93 ± 21 | 261 ± 86* | 102 ± 6 |
| T-Cho (mg/dl) | 99 ± 12* | 66 ± 5 | 97 ± 9* | 71 ± 4 |
| TG (mg/dl) | 304 ± 127* | 26 ± 7 | 377 ± 119* | 24 ± 6 |
| AST (IU/l) | 454 ± 331 | 170 ± 16 | 351 ± 177* | 152 ± 20 |
| ALT (IU/l) | 333 ± 319 | 38 ± 3 | 234 ± 171* | 39 ± 4 |
| γ-GTP (IU/l) | 6 ± 5 | 2 ± 1 | 4 ± 1* | 2 ± 0 |

* $P<0.05$ vs. Lean rats receiving the same treatment. [a] $P<0.05$ vs. control rats of the same genotype. HbA1c, hemoglobin A1c; T-Cho, total cholesterol; TG, triglyceride; AST, aspartate aminotransferase; ALT, alanine aminotransferase; γ-GTP, γ-glutamyl transpeptidase.

Body weight curves are shown in Fig. 2C. The average body weights of the ZDF rats were significantly higher than those of the Lean rats throughout the experiment. BBN treatment had no effect on the body weights of the ZDF and Lean rats. There was no significant difference in the total intake of BBN between ZDF (1.4 ± 0.5 mg/kg b.w.) and Lean rats (1.1 ± 0.0 mg/kg b.w.).

*Time course changes of blood glucose level*

Time course changes of the blood glucose level in experiments 1 and 2 are shown in Fig. 2B and D, respectively.

In experiment 1, the blood glucose levels were significantly higher in the DMBDD-treated and control ZDF rats throughout the experimental period compared with the Lean rats receiving the same treatment, respectively. The level of blood glucose was significantly suppressed only in the DMBDD-treated ZDF rats compared with the control ZDF rats at weeks 3–5, possibly due to the significant decrease of food intake during weeks 1–5 caused by DMBDD treatment.

In experiment 2, the blood glucose levels of the BBN-initiated and control ZDF rats were significantly elevated compared with the Lean rats receiving the same treatment from week 2 to 4; however, BBN treatment had no effect on the blood glucose level in the ZDF and Lean rats.

*Blood biochemistry*

The results of the blood biochemistry analysis in experiments 1 and 2 are shown in Table 2.

In experiment 1, the level of hemoglobin A1c (HbA1c),

**Table 3.** Histopathological Findings (Experiment 1)

| Site and type of lesion | | Incidence (%) | | | | Multiplicity (No./rat) | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | DMBDD | | Control | | DMBDD | | Control | |
| | | ZDF | Lean | ZDF | Lean | ZDF | Lean | ZDF | Lean |
| Effective no. of rats [b] | | 20 | 20 | 6 | 7 | 20 | 20 | 6 | 7 |
| Urinary bladder | Simple hyperplasia | 19 (95)[a] | 14 (70)[a] | 0 (0) | 0 (0) | - | - | - | - |
| | PN hyperplasia | 9 (45) | 6 (30) | 0 (0) | 0 (0) | 1.0 ± 1.3[a] | 0.4 ± 0.7[a] | 0 | 0 |
| | Papilloma | 4 (20) | 4 (20) | 0 (0) | 0 (0) | 0.3 ± 0.7 | 0.3 ± 0.6 | 0 | 0 |
| | TCC | 11 (55)*, [a] | 1 (5) | 0 (0) | 0 (0) | 0.7 ± 0.8*, [a] | 0.1 ± 0.2 | 0 | 0 |
| | Total tumors [c] | 14 (70)*, [a] | 5 (25) | 0 (0) | 0 (0) | 1.0 ± 0.9*, [a] | 0.3 ± 0.6[a] | 0 | 0 |
| Colon | Adenoma | 1 (5) | 0 (0) | 0 (0) | 0 (0) | 0.1 ± 0.2 | 0 | 0 | 0 |
| | Adenocarcinoma | 10 (50)* | 3 (15) | 0 (0) | 0 (0) | 0.8 ± 0.9*, [a] | 0.2 ± 0.5 | 0 | 0 |
| | Mucinous carcinoma | 3 (15) | 1 (5) | 0 (0) | 0 (0) | 0.2 ± 0.4 | 0.1 ± 0.2 | 0 | 0 |
| | Total tumors | 12 (60)*, [a] | 4 (20) | 0 (0) | 0 (0) | 1.0 ± 1.0*, [a] | 0.3 ± 0.6 | 0 | 0 |
| Small intestine | Adenocarcinoma | 9 (45)* | 0 (0) | 0 (0) | 0 (0) | 0.7 ± 1.0* | 0 | 0 | 0 |
| | Mucinous carcinoma | 1 (5) | 0 (0) | 0 (0) | 0 (0) | 0.1 ± 0.2 | 0 | 0 | 0 |
| | Total tumors | 10 (50)* | 0 (0) | 0 (0) | 0 (0) | 0.8 ± 1.0*, [a] | 0 | 0 | 0 |
| Liver | Adenoma | 8 (40)* | 1 (5) | 0 (0) | 0 (0) | 0.6 ± 0.8*, [a] | 0.1 ± 0.2 | 0 | 0 |
| Kidney | Adenoma | 4 (20) | 7 (35) | 0 (0) | 0 (0) | 0.2 ± 0.4[a] | 0.5 ± 0.7[a] | 0 | 0 |
| | Mesenchymal tumor | 5 (25) | 2 (10) | 0 (0) | 0 (0) | 0.3 ± 0.4[a] | 0.1 ± 0.3 | 0 | 0 |
| | Nephroblastoma | 9 (45) | 3 (15) | 0 (0) | 0 (0) | 0.7 ± 0.9*, [a] | 0.2 ± 0.4 | 0 | 0 |
| | TCC | 2 (10) | 0 (0) | 0 (0) | 0 (0) | 0.2 ± 0.5 | 0 | 0 | 0 |
| | Total tumors | 12 (60)[a] | 9 (45) | 0 (0) | 0 (0) | 1.3 ± 1.2[a] | 0.7 ± 0.9[a] | 0 | 0 |
| Lung | Adenoma | 5 (25)* | 20 (100)[a] | 0 (0) | 0 (0) | 0.4 ± 0.7*, [a] | 3.7 ± 2.0[a] | 0 | 0 |
| | Carcinoma | 3 (15) | 3 (15) | 0 (0) | 0 (0) | 0.2 ± 0.5 | 0.3 ± 0.8 | 0 | 0 |
| | Total tumors | 7 (35)* | 20 (100)[a] | 0 (0) | 0 (0) | 0.6 ± 0.8*, [a] | 4.0 ± 2.4[a] | 0 | 0 |
| Thyroid gland | Follicular adenoma | 1 (5) | 2 (10) | 0 (0) | 0 (0) | 0.1 ± 0.4 | 0.1 ± 0.3 | 0 | 0 |
| | Follicular carcinoma | 0 (0)* | 5 (25) | 0 (0) | 0 (0) | 0* | 0.3 ± 0.4[a] | 0 | 0 |
| | Total tumors | 1 (5)* | 7 (35) | 0 (0) | 0 (0) | 0.1 ± 0.4 | 0.4 ± 0.5[a] | 0 | 0 |
| Esophagus | Papilloma | 3 (15) | 0 (0) | 0 (0) | 0 (0) | 0.2 ± 0.4 | 0 | 0 | 0 |
| Forestomach | SCC | 1 (5) | 0 (0) | 0 (0) | 0 (0) | 0.1 ± 0.2 | 0 | 0 | 0 |

* $P<0.05$ vs. Lean rats receiving the same treatment. [a] $P<0.05$ vs. control rats of the same genotype. PN, papillary or nodular; TCC, transitional cell carcinoma; SCC, squamous cell carcinoma. [b] Number of rats found dead or moribund from week 17 to 30. [c] Papilloma + TCC.

one of the characteristics of diabetes, was significantly higher in both the DMBDD-treated and control ZDF animals than in the Lean rats receiving the same treatment. Furthermore, the total cholesterol (T-Cho) and triglyceride (TG) levels were also significantly increased in the DMBDD-administered and control ZDF rats but not in the Lean rats receiving the same treatment.

In experiment 2, the levels of HbA1c, T-Cho and TG were significantly higher in both the BBN-treated and control ZDF rats than in the Lean rats receiving the same treatment. Moreover, no influence of BBN treatment was apparent on HbA1c, T-Cho and TG in both the ZDF and Lean rats.

*Histopathological findings in experiment 1*

Table 3 summarizes the data on the incidence and multiplicity of preneoplastic and neoplastic lesions induced by DMBDD administration. No tumors were found in the control ZDF and Lean rats. Macroscopically, several very large tumors occupying the whole urinary bladder were found in the DMBDD-treated ZDF rats but not in the Lean rats (Fig. 3). Histological examination demonstrated significant elevation of the incidences and multiplicities of transitional cell carcinomas (TCC: 55%, 0.7 ± 0.8/rat) and total tumors (papilloma +TCC: 70%, 1.0 ± 0.9/rat) in the DMBDD-administered ZDF animals as compared with the DMBDD-initiated Lean rats (TCC, 5% and 0.1 ± 0.2/rat; total tumors, 25% and 0.3 ± 0.6/rat).

The incidences and multiplicities of colon adenocarcinoma and total colon tumors (adenocarcinoma, 50% and 0.8 ± 0.9/rat; total tumors, 60% and 1.0 ± 1.0/rat) were significantly increased in the DMBDD-treated ZDF rats as compared with the DMBDD-treated Lean rats (adenocarcinoma, 15% and 0.2 ± 0.5/rat; total tumors, 20% and 0.3 ± 0.6/rat). Furthermore, the incidences and multiplicities of small intestine adenocarcinoma and total tumors were significantly higher in the DMBDD-administered ZDF rats (adenocarcinoma, 45% and 0.7 ± 1.0/rat; total tumors, 50% and 0.8 ± 1.0/rat) than in the DMBDD-treated Lean animals (no tumor was found).

The incidence and multiplicity of hepatocellular adenoma were significantly increased in the DMBDD-treated ZDF rats (40%, 0.6 ± 0.8/rat) compared with the DMBDD-

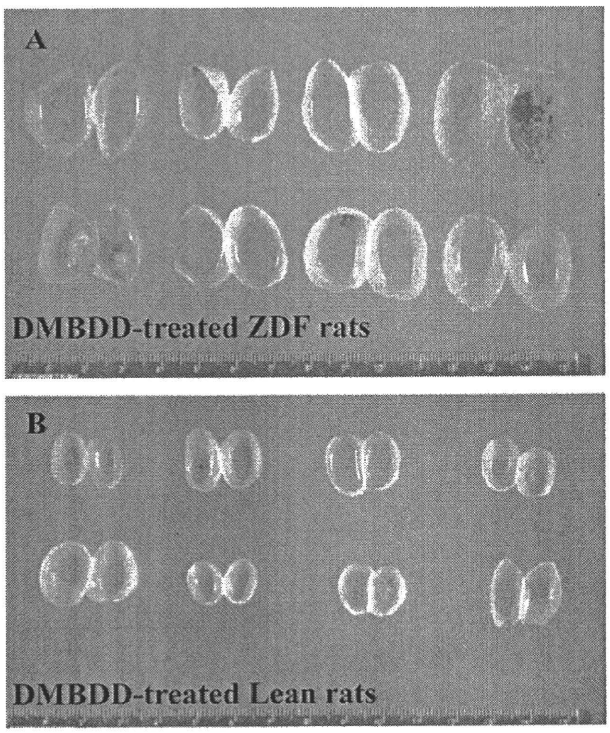


**Fig. 3.** Macroscopic view of the urinary bladder in experiment 1. A: DMBDD-treated ZDF rats. B: DMBDD-treated Lean rats.

treated Lean group (5%, 0.1 ± 0.2/rat). No hepatocellular carcinomas were found in the DMBDD-initiated ZDF and Lean rats.

No renal cell carcinoma was observed in any groups. There were no significant differences in renal cell adenoma between the DMBDD-treated ZDF and Lean rats. The multiplicity of nephroblastoma in the DMBDD-treated ZDF rats (0.7 ± 0.9/rat) was significantly increased compared with the DMBDD-administered Lean animals (0.2 ± 0.4/rat).

On the other hand, the incidences and multiplicities of adenoma and total tumors in lung and thyroid follicular carcinoma were significantly decreased in the DMBDD-treated ZDF rats compared with the DMBDD-initiated Lean group.

*Serum levels of insulin, leptin, adiponectin and IGF-1*

In experiment 1, the serum insulin level in the DMBDD-treated ZDF rats was significantly higher than in the treated Lean groups (Fig. 4A, $P<0.001$). Furthermore, a significant increase in serum leptin level was found in the ZDF rats compared with the Lean rats receiving the same treatment (Fig 4C; DMBDD-treated, $P<0.0001$; control, $P<0.05$). Moreover, there was a significant decrease in the serum adiponectin level in the ZDF rats compared with the Lean rats receiving the same treatment (Fig. 4E; DMBDD-treated, $P<0.0001$; control, $P<0.05$). In contrast to our expectation, the serum levels of IGF-1 of the DMBDD-treated and control ZDF rats were significantly decreased compared with the Lean rats receiving the same treatment (Fig. 4G; DMBDD-treated, $P<0.0001$; control, $P<0.0001$).

In experiment 2, the serum insulin level was significantly increased in the BBN-treated and control ZDF rats compared with the Lean rats receiving the same treatment (Fig. 4B; BBN-treated, $P<0.0001$; control, $P<0.0001$). Similarly, the serum leptin level in the ZDF rats was significantly increased compared with the Lean rats receiving the same treatment (Fig. 4D; BBN-treated, $P<0.0001$; control, $P<0.0001$); however, no significant differences were apparent in the serum level of adiponectin and IGF-1 (Fig. 4F and H).

The TNFα and IL-6 serum levels were under the limit of detection.

*IGF-1 mRNA expression in the liver*

Since there was no increase of the IGF-1 level in the serum of ZDF rats at weeks 4 and 30, its mRNA expression level was examined in the liver, which is the main organ in which IGF-1 is produced. IGF-1 mRNA expression was inhibited in the liver of the DMBDD-treated and control ZDF rats compared with the Lean rats receiving the same treatment at week 30 in experiment 1 but without significance (Fig. 5A). Furthermore, there was a significant decrease of IGF-1 mRNA expression in the BBN-treated and control ZDF rats compared with the Lean rats receiving the same treatment at week 4 in experiment 2 (Fig. 5B; BBN-treated, $P<0.05$; control, $P<0.001$).

*PI3K, p53 and PCNA mRNA expression in the bladder epithelium of BBN-treated rats (experiment 2)*

Since alterations of the serum levels of insulin, leptin and adiponectin were observed, we further investigated the downstream effector molecules of these receptors.

Significant increases of PI3K mRNA expression in the bladder epithelium of the BBN-initiated and control ZDF rats were found compared with the Lean rats receiving the same treatment (Fig. 6A; BBN-treated, $P<0.0001$; control, $P<0.001$).

Four-week treatment with BBN significantly decreased the p53 mRNA expression level in the urothelium of the ZDF rats but had no effect on the Lean rats (Fig. 6B, $P<0.001$). Furthermore, p53 mRNA expression was significantly inhibited in the BBN-treated ZDF rats compared with the treated Lean animals (Fig. 6B, $P<0.05$).

The expression of mRNA of PCNA, which is a marker for cell proliferation, was significantly increased in the BBN-treated ZDF rats compared with the control ZDF animals (Fig. 6C, $P<0.05$) and showed a tendency to increase as compared with the BBN-treated Lean rats.

## Discussion

The present study demonstrated enhancement effects of T2DM on urinary bladder, colon and liver carcinogenesis in ZDF type 2 diabetes rats, indicating the high multiorgan carcinogenic susceptibility of this strain of rats. In epidemiological studies, T2DM was reported to be associated with

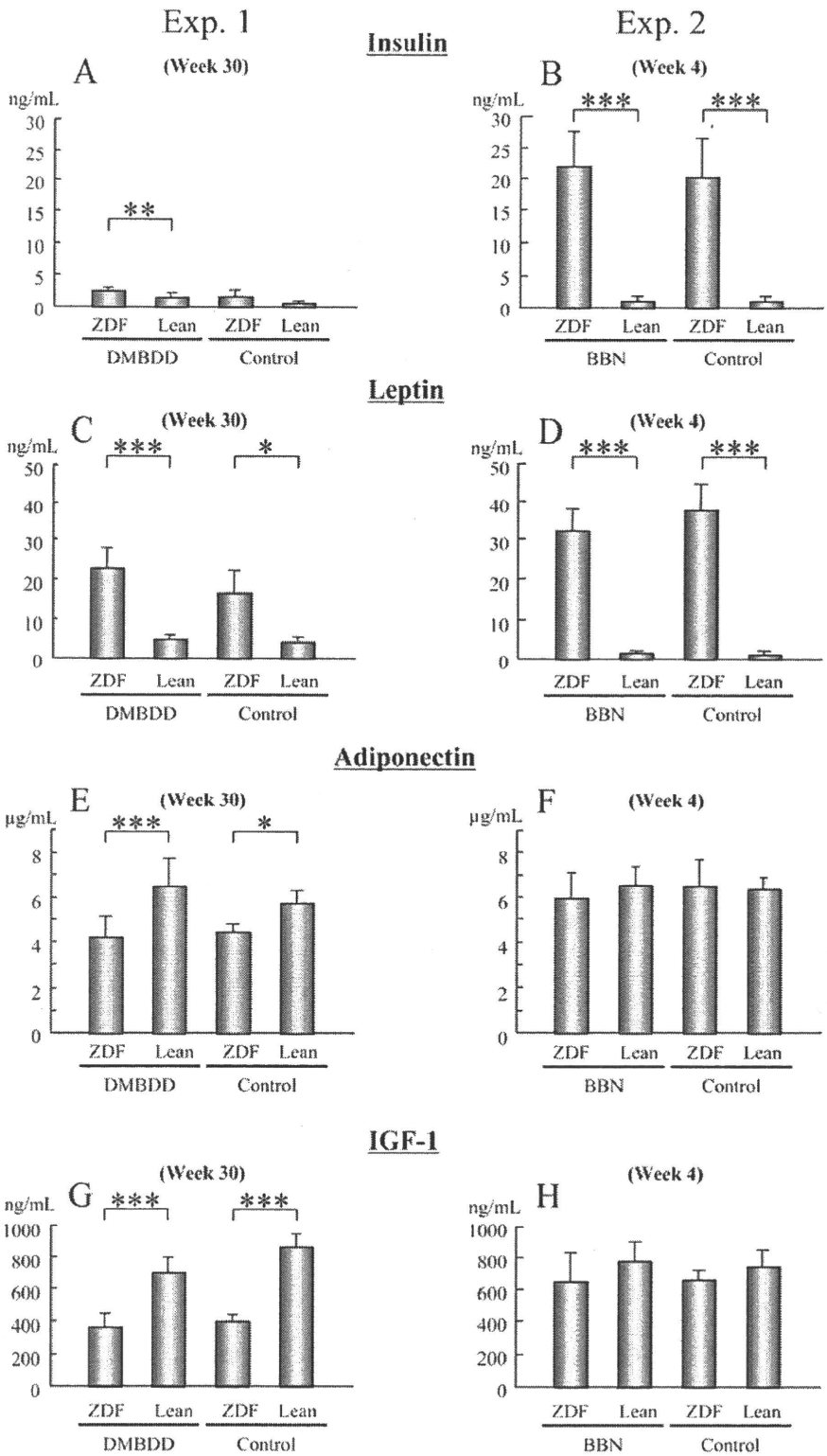


**Fig. 4.** Serum levels of insulin, leptin, adiponectin and IGF-1 in ZDF and Lean rats. Data from experiment 1 (A, C, E and G) and experiment 2 (B, D, F and H). Serum concentrations of insulin (A and B), leptin (C and D), adiponectin (E and F) and IGF-1 (G and H) were measured by ELISA. *, ** and ***: $P<0.05$, $P<0.001$ and $P<0.0001$, respectively, vs. Lean rats receiving the same treatment.

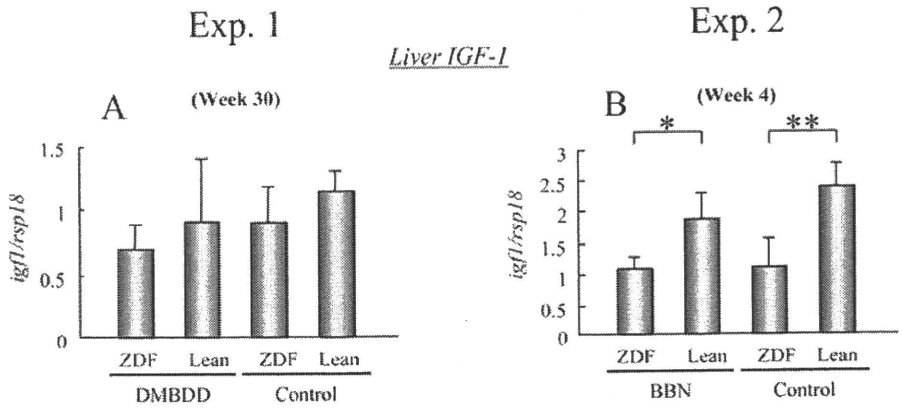


**Fig. 5.** IGF-1 mRNA expression in the liver. * and **: $P<0.05$ and $P<0.001$, respectively, vs. Lean rats receiving the same treatment.

elevation of colon, pancreas, mammary, liver and urinary bladder cancers[2,3]. To the best of our knowledge, this study provides the first experimental evidence for a relationship between DM and urinary bladder cancer.

Several serum changes in T2DM patients have been suggested to be responsible for the increased cancer risks, including increases in insulin, IGF-1, leptin, TNFα and IL-6, as well as decreased adiponectin[15]. In the present study, the serum insulin level was significantly higher in the DMBDD-treated ZDF rats than in the DMBDD-treated Lean rats at week 30. It has been reported that hyperinsulinemia by injection of insulin enhanced the tumorigenesis of azoxymethane-induced colon carcinogenesis in rats[16,17]. Furthermore, hyperinsulinemia is considered to promote carcinogenesis not only directly but also indirectly by increasing the synthesis of IGF-1[18]. Unexpectedly, the serum level of IGF-1 was significantly lower in the ZDF rats than in the Lean rats irrespective of whether or not they received carcinogen treatment at week 30, and there was no significant difference at week 4 between the two strains of rats. In contrast, the IGF-1 mRNA expression level in the liver, the major site of IGF-1 synthesis, was significantly lower in the ZDF rats than in the Lean rats receiving the same treatment at week 4 but was not significant at week 30 between the two strains of rats. Further study examining the protein expression level of IGF-1 in the liver to explain this discrepancy is necessary. Nevertheless, the changes of serum IGF-1 were not related to the increased cancers of the urinary bladder, colon and liver under the present conditions.

In recent studies, leptin, a hormone secreted by adipocytes, was indicated to act as a mitogen and angiogenic factor in addition to its neuroendocrine function[19]. Furthermore, epidemiological studies have shown that an elevated level of serum leptin was associated with a high risk of colorectal cancer in men[20] and breast cancer[21]. Meanwhile, adiponectin, which is also an adipocyte-secreted hormone, was reported to have an antiproliferative effect[22]; thus, low adiponectin concentrations were associated with malignancies in the colon[23] or mammary gland[24]. In the present study, neither DMBDD initiation nor BBN treatment affected the serum levels of leptin or adiponectin in the ZDF and Lean rats; however, the serum level of leptin was significantly higher and the serum adiponectin level was significantly lower in the ZDF rats than in the Lean rats receiving the same treatment at week 30. Therefore, the elevation of leptin and decrease of adiponectin may represent a highly susceptible risk of carcinogenesis of the urinary bladder, colon and liver.

Recently, epidemiological studies have clearly indicated that individuals with diabetes have an increased risk of bladder cancer[3]; however, the mechanisms by which diabetes mellitus contributes to bladder carcinoma are not clear. In this study, both the incidence and multiplicity of bladder cancer were significantly increased in the DMBDD-treated ZDF rats compared with the treated Lean rats in the 30-week multiorgan carcinogenicity study. In the 4-week BBN bladder carcinogenicity study, the serum insulin level at week 4 was significantly increased in the treated and control ZDF rats compared with the Lean rats receiving the same treatment. Similarly, the serum leptin level in the ZDF rats was significantly increased compared with the Lean rats receiving the same treatment at week 4. On the other hand, no significant differences were apparent in the serum adiponectin level at week 4. Thus, changes of insulin and leptin, but not adiponectin levels, may be related to the high susceptibility of ZDF rats to bladder carcinogenesis from an early stage of carcinogenesis. Insulin and leptin have been reported to activate the PI3K signaling pathway[15], which is known to be associated with malignant behavior, including cell growth, proliferation and cell survival[25]. Furthermore, its activation of PI3K has been demonstrated to be responsible for carcinogenesis in regard to bladder, colon, liver and other cancers[26–29]. High mRNA expression levels of PI3K in the ZDF rats suggested that the PI3K pathway may be responsible for the high susceptibility to bladder carcinogenesis of rats with T2DM.

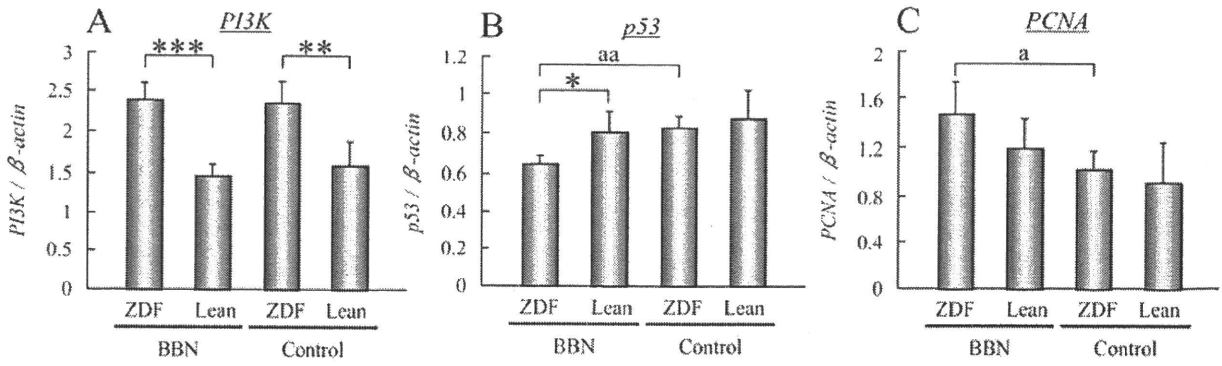


**Fig. 6.** PI3K, p53 and PCNA mRNA expression in the bladder epithelium of BBN-treated rats (experiment 2). *, ** and ***: $P<0.05$, $P<0.001$ and $P<0.0001$, respectively, vs. Lean rats receiving the same treatment. a and aa: $P<0.05$ and $P<0.001$, respectively, vs. control rats of the same genotype.

In addition, the decrease of p53 in obesity has been demonstrated to play an important role in obesity-associated cancers, such as breast and prostate cancer[30,31]. The low mRNA expression level of p53 in the BBN-treated ZDF rats compared with the BBN-treated Lean rats may be responsible, at least in part, for their high susceptibility to bladder carcinogenesis.

No increased risk of lung cancer in diabetes was found in a recent epidemiological study[32]. With regard to the relationship between diabetes and thyroid cancer risk, there is no report available in the published literature. The findings of the present study that the incidences and multiplicities of lung tumors and thyroid follicular carcinoma were significantly decreased in the DMBDD-treated ZDF rats compared with the DMBDD-initiated Lean group indicate a low susceptibility of ZDF rats to lung and thyroid carcinogenesis in this model. However, the mechanism by which T2DM affects lung and thyroid carcinogenesis and whether these findings are relevant to human lung and thyroid cancers are not currently clear.

In conclusion, our results demonstrated that urinary bladder, colon and liver carcinogenesis are enhanced in ZDF type 2 diabetes rats. The possible mechanisms are related to increased serum levels of insulin and leptin and a decreased serum level of adiponectin. Furthermore, the high susceptibility to bladder carcinogenesis in T2DM might be a consequence of PI3K pathway activation and decreased p53 expression. Further studies to analyze the mechanisms underlying the high susceptibility of T2DM rats to colon and liver carcinogenesis are necessary.

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## References

1. Kasper DL, Brouwaid E, Fauci AS, Hauser SL, Longo DL, and Jameson JL. HARRISON'S PRINCIPLES OF Internal Medicine, Vol. 2005.
2. Nicolucci A. Epidemiological aspects of neoplasms in diabetes. Acta Diabetol **47**: 87–95. 2010.[Medline] [CrossRef]
3. Larsson SC, Orsini N, Brismar K, and Wolk A. Diabetes mellitus and risk of bladder cancer: a meta-analysis. Diabetologia. **49**: 2819–2823. 2006.[Medline] [CrossRef]
4. Terai K, Sakamoto K, Goto M, Matsuda M, Kasamaki S, Shinmura K, Takita N, and Kamano T. Greater development of 1,2-dimethylhydrazine-induced colon cancer in a rat model of type 2 diabetes mellitus. J Int Med Res. **34**: 385–389. 2006.[Medline]
5. Yoshizawa N, Yamaguchi H, Yamamoto M, Shimizu N, Furihata C, Tatematsu M, Seto Y, and Kaminishi M. Gastric carcinogenesis by N-Methyl-N-nitrosourea is enhanced in db/db diabetic mice. Cancer Sci. **100**: 1180–1185. 2009.[Medline] [CrossRef]
6. Cocca C, Gutierrez A, Nunez M, Croci M, Martin G, Cricco G, Rivera E, and Bergoc R. Suppression of mammary gland tumorigenesis in diabetic rats. Cancer Detect Prev. **27**: 37–46. 2003.[Medline] [CrossRef]
7. Giovannucci E. Insulin and colon cancer. Cancer Causes Control. **6**: 164–179. 1995.[Medline] [CrossRef]
8. Clark JB, Palmer CJ, and Shaw WN. The diabetic Zucker fatty rat. Proc Soc Exp Biol Med. **173**: 68–75. 1983.[Medline]
9. Friedman JE, de Vente JE, Peterson RG, and Dohm GL. Altered expression of muscle glucose transporter GLUT-4 in diabetic fatty Zucker rats (ZDF/Drt-fa). Am J Physiol. **261**: E782–E788. 1991.[Medline]
10. Sturis J, Pugh WL, Tang J, Ostrega DM, Polonsky JS, and Polonsky KS. Alterations in pulsatile insulin secretion in the Zucker diabetic fatty rat. Am J Physiol. **267**: E250–E259. 1994.[Medline]
11. Fukushima S, Hagiwara A, Hirose M, Yamaguchi S, Ti-

wawech D, and Ito N. Modifying effects of various chemicals on preneoplastic and neoplastic lesion development in a wide-spectrum organ carcinogenesis model using F344 rats. Jpn J Cancer Res. **82**: 642–649. 1991.[Medline]

12. Doi K, Wanibuchi H, Salim EI, Shen J, Wei M, Mitsuhashi M, Kudoh S, Hirata K, and Fukushima S. Revised rat multi-organ carcinogenesis bioassay for whole-body detection of chemopreventive agents: modifying potential of S-methylcysteine. Cancer Lett. **206**: 15–26. 2004.[Medline] [CrossRef]
13. Wei M, Arnold L, Cano M, and Cohen SM. Effects of co-administration of antioxidants and arsenicals on the rat urinary bladder epithelium. Toxicol Sci. **83**: 237–245. 2005.[Medline] [CrossRef]
14. Wei M, Hamoud AS, Yamaguchi T, Kakehashi A, Morimura K, Doi K, Kushida M, Kitano M, Wanibuchi H, and Fukushima S. Potassium bromate enhances N-ethyl-N-hydroxyethylnitrosamine-induced kidney carcinogenesis only at high doses in Wistar rats: indication of the existence of an enhancement threshold. Toxicol Pathol. **37**: 983–991. 2009.[Medline] [CrossRef]
15. Huang XF, and Chen JZ. Obesity, the PI3K/Akt signal pathway and colon cancer. Obes Rev. **10**: 610–616. 2009.[Medline] [CrossRef]
16. Corpet DE, Jacquinet C, Peiffer G, and Tache S. Insulin injections promote the growth of aberrant crypt foci in the colon of rats. Nutr Cancer. **27**: 316–320. 1997.[Medline] [CrossRef]
17. Tran TT, Medline A, and Bruce WR. Insulin promotion of colon tumors in rats. Cancer Epidemiol Biomarkers Prev. **5**: 1013–1015. 1996.[Medline]
18. Boni-Schnetzler M, Schmid C, Meier PJ, and Froesch ER. Insulin regulates insulin-like growth factor I mRNA in rat hepatocytes. Am J Physiol. **260**: E846–E851. 1991.[Medline]
19. Garofalo C and Surmacz E. Leptin and cancer. J Cell Physiol. **207**: 12–22. 2006.[Medline] [CrossRef]
20. Stattin P, Palmqvist R, Soderberg S, Biessy C, Ardnor B, Hallmans G, Kaaks R, and Olsson T. Plasma leptin and colorectal cancer risk: a prospective study in Northern Sweden. Oncol Rep. **10**: 2015–2021. 2003.[Medline]
21. Han C, Zhang HT, Du L, Liu X, Jing J, Zhao X, Yang X, and Tian B. Serum levels of leptin, insulin, and lipids in relation to breast cancer in china. Endocrine. **26**: 19–24. 2005.[Medline] [CrossRef]
22. Barb D, Williams CJ, Neuwirth AK, and Mantzoros CS. Adiponectin in relation to malignancies: a review of existing basic research and clinical evidence. Am J Clin Nutr. **86**: s858–s866. 2007.[Medline]
23. Wei EK, Giovannucci E, Fuchs CS, Willett WC, and Mantzoros CS. Low plasma adiponectin levels and risk of colorectal cancer in men: a prospective study. J Natl Cancer Inst. **97**: 1688–1694. 2005.[Medline] [CrossRef]
24. Tworoger SS, Eliassen AH, Kelesidis T, Colditz GA, Willett WC, Mantzoros CS, and Hankinson SE. Plasma adiponectin concentrations and risk of incident breast cancer. J Clin Endocrinol Metab. **92**: 1510–1516. 2007.[Medline] [CrossRef]
25. Cantley LC. The phosphoinositide 3-kinase pathway. Science. **296**: 1655–1657. 2002.[Medline] [CrossRef]
26. Knowles MA, Platt FM, Ross RL, and Hurst CD. Phosphatidylinositol 3-kinase (PI3K) pathway activation in bladder cancer. Cancer Metastasis Rev. **28**: 305–316. 2009.[Medline] [CrossRef]
27. Philp AJ, Campbell IG, Leet C, Vincan E, Rockman SP, Whitehead RH, Thomas RJ, and Phillips WA. The phosphatidylinositol 3'-kinase p85alpha gene is an oncogene in human ovarian and colon tumors. Cancer Res. **61**: 7426–7429. 2001.[Medline]
28. Yeh KT, Chang JG, Chen YJ, Chen ST, Yu SY, Shih MC, Perng LI, Wang JC, Tsai M, and Chang CP. Mutation analysis of the putative tumor suppressor gene PTEN/MMAC1 in hepatocellular carcinoma. Cancer Invest. **18**: 123–129. 2000.[Medline] [CrossRef]
29. Vivanco I, and Sawyers CL. The phosphatidylinositol 3-kinase AKT pathway in human cancer. Nat Rev Cancer. **2**: 489–501. 2002.[Medline] [CrossRef]
30. Chen C, Chang YC, Liu CL, Chang KJ, and Guo IC. Leptin-induced growth of human ZR-75–1 breast cancer cells is associated with up-regulation of cyclin D1 and c-Myc and down-regulation of tumor suppressor p53 and p21WAF1/CIP1. Breast Cancer Res Treat. **98**: 121–132. 2006.[Medline] [CrossRef]
31. Mistry T, Digby JE, Desai KM, and Randeva HS. Leptin and adiponectin interact in the regulation of prostate cancer cell growth via modulation of p53 and bcl-2 expression. BJU Int. **101**: 1317–1322. 2008.[Medline] [CrossRef]
32. Hall GC, Roberts CM, Boulis M, Mo J, and MacRae KD. Diabetes and the risk of lung cancer. Diabetes Care. **28**: 590–594. 2005.[Medline] [CrossRef]

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Research Article

Cancer Prevention Research

# Acyclic Retinoid Inhibits Diethylnitrosamine-Induced Liver Tumorigenesis in Obese and Diabetic C57BLKS/J- +Lepr$^{db}$/+Lepr$^{db}$ Mice

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## Abstract

Obesity and the related metabolic abnormalities are associated with increased risk of hepatocellular carcinoma (HCC). Malfunctioning of retinoid X receptor (RXR) α due to phosphorylation by Ras/MAPK also plays a critical role in liver carcinogenesis. In the present study, we examined the effects of acyclic retinoid (ACR), which targets RXRα, on the development of diethylnitrosamine (DEN)-induced liver tumorigenesis in C57BLKS/J- +Lepr$^{db}$/+Lepr$^{db}$ (*db/db*) obese mice. Male *db/db* mice were given tap water containing 40 ppm DEN for 2 weeks, after which they were fed a diet containing 0.03% or 0.06% of ACR throughout the experiment. In mice treated with either dose of ACR for 34 weeks, the development of liver cell adenomas was significantly inhibited as compared with basal diet-fed mice. ACR markedly inhibited the activation of Ras and phosphorylation of the ERK (extracellular signal-regulated kinase) and RXRα proteins in the livers of experimental mice. It also increased the expression of *RARβ* and *p21$^{CIP1}$* mRNA while decreasing the expression of *cyclin D1*, *c-Fos*, and *c-Jun* mRNA in the liver, thereby restoring RXRα function. Administration of ACR improved liver steatosis and activated the AMPK protein. The serum levels of insulin decreased by ACR treatment, whereas the quantitative insulin sensitivity check index (QUICKI) values increased, indicating improved insulin sensitivity. The serum levels of TNF-α and the expression levels of *TNF-α*, *IL-6*, and *IL-1β* mRNA in the livers of DEN-treated *db/db* mice were decreased by ACR treatment, suggesting attenuation of the chronic inflammation induced by excessive fatty deposits. ACR may be, therefore, useful in the chemoprevention of obesity-related HCC. *Cancer Prev Res; 4(1); 128–36. ©2010 AACR.*

## Introduction

Hepatocellular carcinoma (HCC) is a serious health-care problem worldwide. The risk factors associated with the development of HCC include chronic hepatitis B and/or hepatitis C infection, particularly with subsequent cirrhosis. Recent evidence also indicates that obesity and the related metabolic abnormalities, especially diabetes mellitus, increase the risk of HCC (1–3). In a rodent model, the occurrence of diethylnitrosamine (DEN)-induced liver tumorigenesis was found to be significantly higher in obese and diabetic C57BLKS/J- +Lepr$^{db}$/+Lepr$^{db}$ (*db/db*) mice than in genetic control mice (4). Diabetes mellitus has been shown to increase the risk of primary HCC in patients with viral hepatitis (5). Insulin resistance is also significantly associated with the recurrence of stage I HCC after curative treatment (6). Nonalcoholic fatty liver disease (NAFLD) is a hepatic manifestation of the insulin resistance syndrome, and in a subset of NAFLD patients, the condition progresses to nonalcoholic steatohepatitis, which involves severe inflammation and therefore poses the threat of HCC (7, 8). Coexistent obesity or steatosis exacerbates liver injury and fibrosis and thus is involved in liver tumorigenesis (9). Therefore, patients with obesity and insulin resistance comprise a high-risk group for HCC, and their treatment must target the prevention of this malignancy.

Acyclic retinoid (ACR, the same substance as NIK-333), a synthetic retinoid, apparently exerts chemopreventive effects on the development of HCC (10). It inhibits experimental liver carcinogenesis and suppresses the

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growth of HCC-derived cells by inducing apoptosis and causing cell-cycle arrest in $G_0$–$G_1$ (11–15). These effects of ACR are associated with its agonistic activity for distinct nuclear retinoid receptors—retinoid X receptors (RXR) and retinoic acid receptors (RAR), both of which have 3 subtypes (α, β, and γ; 16)—and subsequent expression of the ACR target genes *RARβ* and $p21^{CIP1}$ (12–15). A clinical trial revealed that oral administration of ACR significantly reduced the incidence of posttherapeutic HCC recurrence and improved the survival rates of patients (17, 18). A phase II/III trial of ACR confirmed its effectiveness in preventing second primary HCC in hepatitis C virus–positive patients in a large-scale ($n$ = 401) randomized, placebo-controlled trial; hazard ratio for recurrence-free survival with ACR 600 mg/d versus placebo was 0.27 (95% CI, 0.07–0.96) after 2 years randomization (19).

Among the retinoid receptors, RXRα is considered as one of the most important receptors with respect to the regulation of fundamental cell activities because it forms a heterodimer with other nuclear receptors and thereby acts as the master regulator of nuclear receptors (20). Recent studies indicate that phosphorylation of RXRα abolishes its ability to form a heterodimer with RARβ, and the accumulation of phosphorylated RXRα (p-RXRα, i.e., nonfunctional RXRα), which is caused by activation of the Ras/mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) signaling pathway, plays a critical role in the development of HCC (10, 21, 22). On the other hand, the effects of ACR in suppressing growth and inducing apoptosis in HCC cells depend on the inactivation of Ras-ERK signaling system and subsequent RXRα dephosphorylation (15, 23, 24). In the present study, we examined the effects of ACR on obesity-related liver tumorigenesis by focusing on the inhibition of RXRα phosphorylation. We also examined whether ACR treatment improves the insulin resistance, liver steatosis, and inflammatory condition caused by obesity with DEN-treated *db/db* mice, a useful preclinical model, to evaluate the mechanisms underlying the inhibition of obesity-related liver tumorigenesis by chemopreventive drugs (4).

## Materials and Methods

### Animals and chemicals

Four-week-old male *db/db* mice were obtained from Japan SLC, Inc. All mice received humane care and were housed at Gifu University Life Science Research Center in accordance with the Institutional Animal Care Guidelines. DEN was purchased from Sigma Chemical Co. ACR was supplied by Kowa Pharmaceutical Co.

### Experimental procedure

The experimental protocol, which was approved by the Institutional Committee of Animal Experiments of Gifu University, was as described previously (4). At 5 weeks of age, 40 *db/db* mice were randomly divided into 5 groups. All the mice in groups 1 ($n$ = 10), 2 ($n$ = 10), and 3 ($n$ = 10) were given tap water containing 40 ppm of DEN for the first 2 weeks, which is sufficient to develop liver neoplasms in *db/db* mice (4). After DEN treatment, the mice in groups 2 and 3 were fed the basal diet CRF-1 (Oriental Yeast Co.) containing 0.03% ACR (group 2) or 0.06% ACR (group 3), respectively, with free access to the feed till the end of experiment. Group 4 ($n$ = 5) was fed the CRF-1 diet containing 0.06% ACR. The mice in groups 1 and 5 ($n$ = 5) were fed the CRF-1 diet throughout the experiment. The rationale for the doses (0.03% and 0.06%) selection of ACR was based on previous studies, in which similar doses of ACR inhibited experimental liver carcinogenesis induced by chemical agents (25, 26). At 41 weeks of age (after 34 weeks of ACR treatment), all the mice were sacrificed by $CO_2$ asphyxiation to check for the development of HCC, liver cell adenoma, and foci of cellular alteration (FCA).

### Histopathologic analysis

At sacrifice, the livers were immediately removed and macroscopically inspected for the presence of neoplasms. Maximum sagittal sections of each lobe (6 lobes) were used for histopathologic examination. For all experimental groups, 4-μm thick sections of formalin-fixed, paraffin-embedded livers were stained routinely with hematoxylin and eosin (H&E) for histopathologic examination. The presence of HCC, liver cell adenoma, and FCA was judged according to previously described criteria (27). The multiplicity of FCA was assessed on a per unit area ($cm^2$) basis.

### Ras activation assay

Ras activity was determined using a Ras activation assay kit (Upstate Biotechnology) according to the manufacturer's instructions. Ras was precipitated in equivalent amounts of liver extract (50 μg) from DEN-treated mice (groups 1–3) by using Raf-1/Ras-binding domain-immobilized agarose, which was then subjected to Western blot analysis using anti-Ras antibody (24). The intensity of the blots was quantified using NIH imaging software Version 1.62.

### Protein extraction and Western blot analysis

Total protein was extracted from the nontumor site of livers of DEN-treated mice, and equivalent amounts of proteins (30 μg per lane) were examined by Western blot analysis (4). Previously described primary antibodies for RXRα (ΔN-197 and D-20), ERK, phosphorylated ERK (p-ERK), Stat3, p-Stat3, AMP-activated kinase (AMPK), p-AMPK, and GAPDH were used (15, 22, 28, 29). The ΔN-197 antibody is considered a specific antibody for the p-RXRα protein (22, 23). The GAPDH antibody served as a loading control.

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### RNA extraction and quantitative real-time reverse transcription PCR

Total RNA was isolated from the nontumor site livers of DEN-treated mice by using the RNAqueous-4PCR kit (Ambion Applied Biosystems). cDNA was amplified from 0.2 μg of total RNA by using the SuperScript III First-Strand Synthesis System (Invitrogen), and quantitative real-time reverse transcription PCR (RT-PCR) analysis was carried out as described previously (4). The specific primers used for amplification of the *TNF-α*, *IL-6*, *IL-1β*, and *β-actin* genes were as described previously (30). The primers for the amplification of *RARβ*, *p21$^{CIP1}$*, *cyclin D1*, *c-Jun*, and *c-Fos* genes are listed in Supplementary Table S1.

### Clinical chemistry

Before sacrifice, the mice were fasted for 6 hours, and at sacrifice, blood samples were collected for assaying the serum concentrations of insulin, glucose, and TNF-α, which was as described previously (4, 29). The serum TNF-α (Shibayagi) levels were determined using an enzyme immunoassay according to the manufacturer's protocol. Insulin resistance was estimated by determining the quantitative insulin sensitivity check index (QUICKI) as follows: $QUICKI = 1/[\log(I_0) + \log(G_0)]$, where $I_0$ is the fasting insulin level and $G_0$ is the fasting glucose level, which correlates with the glucose clamp method (31).

### Hepatic lipid analysis

Approximately 200 mg of frozen liver was homogenized, and lipids were extracted using Folch's method (32). The levels of triglyceride in the liver were measured using the triglyceride E-test kit (Wako Pure Chemical Co.) according to the manufacturer's protocol. To visualize the intrahepatic lipids, Sudan III staining was conducted using the standard procedure with frozen sections.

### Statistical analysis

The results are presented as the mean ± SD and were analyzed using the GraphPad Instat software program Version 3.05 (GraphPad Software) for Macintosh. Differences among the groups were analyzed by either 1-way ANOVA or, as required, by 2-way ANOVA. When the ANOVA showed a statistically significant effect ($P < 0.05$), each experimental group was compared with the control group by using the Tukey–Kramer multiple comparisons test. The differences were considered significant when the 2-sided $P$ value was less than 0.05.

## Results

### General observations

As shown in Table 1, no significant differences were observed in the body, kidney, and fat weights among the groups at the end of the study. A significant decrease in the liver weight was observed in the ACR-treated groups as compared with the basal diet-fed group ($P < 0.05$ or $P < 0.01$), irrespective of DEN treatment. Histopathologic examination showed the absence of ACR toxicity in the liver, kidney, and spleen (data not shown).

### Effects of ACR on DEN-induced liver tumorigenesis in *db/db* mice

Table 2 summarizes the incidence and multiplicity of liver neoplasms (adenoma and HCC) and FCA in the mice from all groups. FCA developed in the livers of mice from all groups, irrespective of DEN treatment. On the other hand, liver cell adenomas developed only in the DEN-treated *db/db* mice. HCCs also developed in all DEN-treated groups; however, the incidence (10% in each group) wan not high. These findings might be associated with experimental protocol because the duration of the experiments (41 weeks) was sufficient to develop adenoma but not HCC. In mice treated with either dose (0.03% and 0.06%) of ACR, the incidence ($P < 0.01$ in each comparison) and multiplicity of adenoma ($P < 0.05$ or $P < 0.01$) were significantly inhibited compared to ACR-untreated mice. The number of FCA was also significantly decreased by ACR treatment, irrespective of DEN treatment ($P < 0.001$ or $P < 0.05$).

### Effects of ACR on Ras activity and phosphorylation of RXRα, ERK, and Stat3 proteins in the livers of DEN-treated *db/db* mice

ACR prevents the growth of HCC cells by inactivating Ras-ERK and dephosphorylating RXRα, thereby restoring RXRα function (10, 15, 23, 24). Stat3 is also an ACR target for the inhibition of cancer cell growth (28). Therefore, the effects of ACR on the inhibition of Ras activity and phosphorylation of the RXRα, ERK, and Stat3 proteins were examined in this study by using an obesity-related liver tumorigenesis model. As shown in Figure 1A, the activity of Raf-1–bound Ras in the liver was significantly inhibited by treatment with either dose of ACR ($P < 0.01$). The expression levels of the p-ERK and p-RXRα proteins were also decreased by ACR treatment (Fig. 1B), indicating that ACR inhibits the development of obesity-related liver neoplasms, at least in part, by dephosphorylating RXRα and thereby restoring its function. At both doses, ACR also decreased the expression levels of the p-Stat3 protein in the livers of DEN-treated *db/db* mice (Fig. 1B).

### Effects of ACR on the expression levels of RARβ, p21$^{CIP1}$, cyclin D1, c-Fos, and c-Jun mRNA in the livers of DEN-treated *db/db* mice

ACR inhibits the growth of HCC cells by increasing the cellular levels of RARβ and p21$^{CIP1}$ but decreasing the levels of cyclin D1, and these effects might be associated with the restoration of RXRα function (12–15). It also suppresses the growth of cancer cells by inhibiting the activity of AP-1, which comprises the Jun and Fos oncoprotein families (28). Therefore, the effect of ACR on the mRNA levels of these molecules was examined next. As shown in Figure 1C, quantitative real-time RT-PCR analysis indicated that ACR treatment

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

| Group no. | Treatment | No. of mice | Weight, g | | | |
|---|---|---|---|---|---|---|
| | | | Body | Liver | Kidney | Fat[a] |
| 1 | DEN alone | 10 | 71.2 ± 8.8[b] | 4.5 ± 0.8 | 0.9 ± 1.0 | 7.5 ± 2.2 |
| 2 | DEN + 0.03% ACR | 10 | 65.7 ± 7.2 | 3.3 ± 1.1[c] | 0.5 ± 0.1 | 6.0 ± 1.5 |
| 3 | DEN + 0.06% ACR | 10 | 66.0 ± 7.4 | 3.0 ± 0.7[d] | 0.5 ± 0.1 | 5.7 ± 1.3 |
| 4 | 0.06% ACR alone | 5 | 66.0 ± 7.4 | 3.0 ± 0.7[e] | 0.5 ± 0.1 | 5.7 ± 1.3 |
| 5 | Basal diet | 5 | 67.9 ± 7.8 | 4.8 ± 1.0 | 0.6 ± 0.1 | 6.2 ± 1.4 |

[a]White adipose tissue of the periorchis and retroperitoneum.
[b]Mean ± SD.
[c]Significantly different from group 1 by Tukey–Kramer multiple comparison test ($P < 0.05$).
[d]Significantly different from group 1 by Tukey–Kramer multiple comparison test ($P < 0.01$).
[e]Significantly different from group 5 by Tukey–Kramer multiple comparison test ($P < 0.05$).

significantly increased the expression levels of *RARβ* and $p21^{CIP1}$ mRNA, especially *RARβ* mRNA, in the livers of DEN-exposed *db/db* mice ($P < 0.01$). On the other hand, the expression levels of *cyclin D1*, *c-Fos*, and *c-Jun* mRNA were significantly decreased by ACR treatment ($P < 0.01$).

### Effects of ACR on hepatic steatosis and the activation of AMPK in the livers of DEN-treated *db/db* mice

Hepatic steatosis is considered a promoter of the development of HCC (8, 9). Therefore, whether ACR treatment enhances the accumulation of lipids in the liver of experimental mice was examined. Examination of Sudan III–stained sections revealed that ACR treatment significantly improved macrovesicular steatosis in the livers of DEN-treated *db/db* mice (Fig. 2A, top panels). The triglyceride levels in the liver were also significantly decreased in mice treated with ACR at either dose ($P < 0.05$) in comparison with those fed the basal diet (Fig. 2A, bottom graph). Moreover, ACR markedly phosphorylated (activated) the AMPK protein, which is a critical serine/threonine kinase that monitors cellular energy status (33), in the livers of the experimental mice (Fig. 2B).

### Effects of ACR on insulin resistance in DEN-treated *db/db* mice

Insulin resistance plays a critical role in the development of HCC (1–6). Therefore, the effects of ACR on the levels of serum insulin and QUICKI values, which indicate the degree of insulin sensitivity, were examined in DEN-treated *db/db* mice. As shown in Figure 2C, the serum insulin level was decreased ($P < 0.05$) whereas the QUICKI value was increased in mice treated with 0.06% ACR ($P < 0.05$)

**Table 2.** Incidence and multiplicity of hepatic neoplasms and FCA in the experimental mice

| Group no. | Treatment | No. of mice | Incidence | | Multiplicity[a] | | FCA |
|---|---|---|---|---|---|---|---|
| | | | Adenoma | HCC | Adenoma | HCC | (No./cm$^2$) |
| 1 | DEN alone | 10 | 7/10 (70%) | 1/10 (10%) | 1.3 ± 1.2[b] | 0.1 ± 0.3 | 15.1 ± 3.5[c] |
| 2 | DEN + 0.03% ACR | 10 | 1/10 (10%)[d] | 1/10 (10%) | 0.2 ± 0.6[e] | 0.1 ± 0.3 | 6.6 ± 2.5[f] |
| 3 | DEN + 0.06% ACR | 10 | 1/10 (10%)[d] | 1/10 (10%) | 0.1 ± 0.3[g] | 0.1 ± 0.3 | 2.8 ± 1.8[f] |
| 4 | 0.06% ACR alone | 5 | 0/5 (0%) | 0/5 (0%) | 0 | 0 | 3.0 ± 2.8[h] |
| 5 | Basal diet | 5 | 0/5 (0%) | 0/5 (0%) | 0 | 0 | 8.0 ± 1.2 |

[a]Number of neoplasms per mouse.
[b]Mean ± SD.
[c]Significantly different from group 5 by Tukey–Kramer multiple comparison test ($P < 0.001$).
[d]Significantly different from group 1 by Fisher's exact probability test ($P < 0.01$).
[e]Significantly different from group 1 by Tukey–Kramer multiple comparison test ($P < 0.05$).
[f]Significantly different from group 1 by Tukey–Kramer multiple comparison test ($P < 0.001$).
[g]Significantly different from group 1 by Tukey–Kramer multiple comparison test ($P < 0.01$).
[h]Significantly different from group 5 by Tukey–Kramer multiple comparison test ($P < 0.05$).

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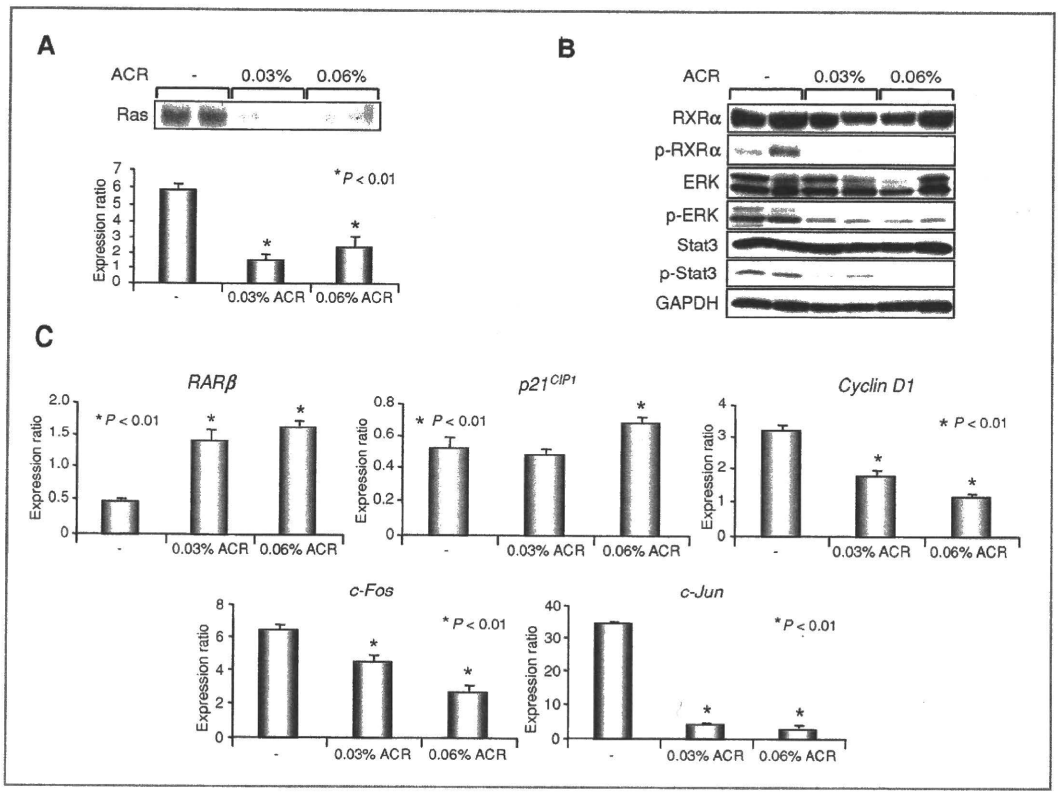


Figure 1. Effects of ACR on Ras activity; phosphorylation of RXRα, ERK, and Stat3 proteins; and the expression of target genes in the livers of DEN-treated *db/db* mice. The total proteins and mRNAs were extracted from the livers of DEN-treated mice. A, the Ras activities were determined using a Ras activation assay kit (top). The relative intensity of the blots was quantified by densitometry and is displayed in the bottom graph. B, the expression levels of the RXRα, p-RXRα, ERK, p-ERK, Stat3, and p-Stat3 proteins were examined by Western blot analysis, using the respective antibodies. Equal protein loading was verified by the detection of GAPDH. Two lanes represent protein samples from two different mice from each group. Repeat Western blots yielded similar results. C, the expression levels of *RARβ*, *p21*$^{CIP1}$, *cyclin D1*, *c-Fos*, and *c-Jun* mRNA were examined by quantitative real-time RT-PCR using specific primers. β-Actin was used as a control. Each experiment was performed in triplicate, and the average value was calculated. Values are the mean ± SD. *, $P < 0.01$ vs. ACR-untreated group.

compared with those in the basal diet–fed group. These findings suggest that ACR improves insulin resistance in obese and diabetic *db/db* mice.

### Effects of ACR on the serum levels of TNF-α and hepatic expression of TNF-α, IL-6, and IL-1β mRNA in DEN-treated *db/db* mice

Because a state of chronic inflammation induced by excessive production of storage lipids and insulin resistance is associated with obesity-related liver carcinogenesis (34), the effects of ACR on the levels of the proinflammatory cytokines TNF-α, IL-6, and IL-1β in DEN-treated *db/db* mice were examined. As shown in Figure 3A, the serum levels of TNF-α were decreased after ACR treatment ($P < 0.01$). Furthermore, the expression levels of *TNF-α*, *IL-6*, and *IL-1β* mRNA in the livers of DEN-treated *db/db* mice were also significantly decreased by ACR treatment ($P < 0.01$). The decrease was most apparent in the levels of *IL-6* mRNA: the inhibition rates were about 85% at both doses of ACR (Fig. 3B).

## Discussion

In the present health care scenario, the effects of obesity, including the promotion of cancer, are critical issues that need to be resolved and HCC is one of the representative malignancies influenced by excessive body weight and related metabolic abnormalities (1–3, 5, 6). A recent clinical trial revealed that supplementation of food with branched-chain amino acids (BCAA), which improves insulin resistance (35), reduced the risk of HCC in obese patients with chronic viral liver disease (3). BCAA supplementation also suppresses liver tumorigenesis in obese and diabetic *db/db* mice by improving insulin resistance and attenuating liver steatosis and fibrosis (4). The results of the present study clearly indicated that ACR also effectively

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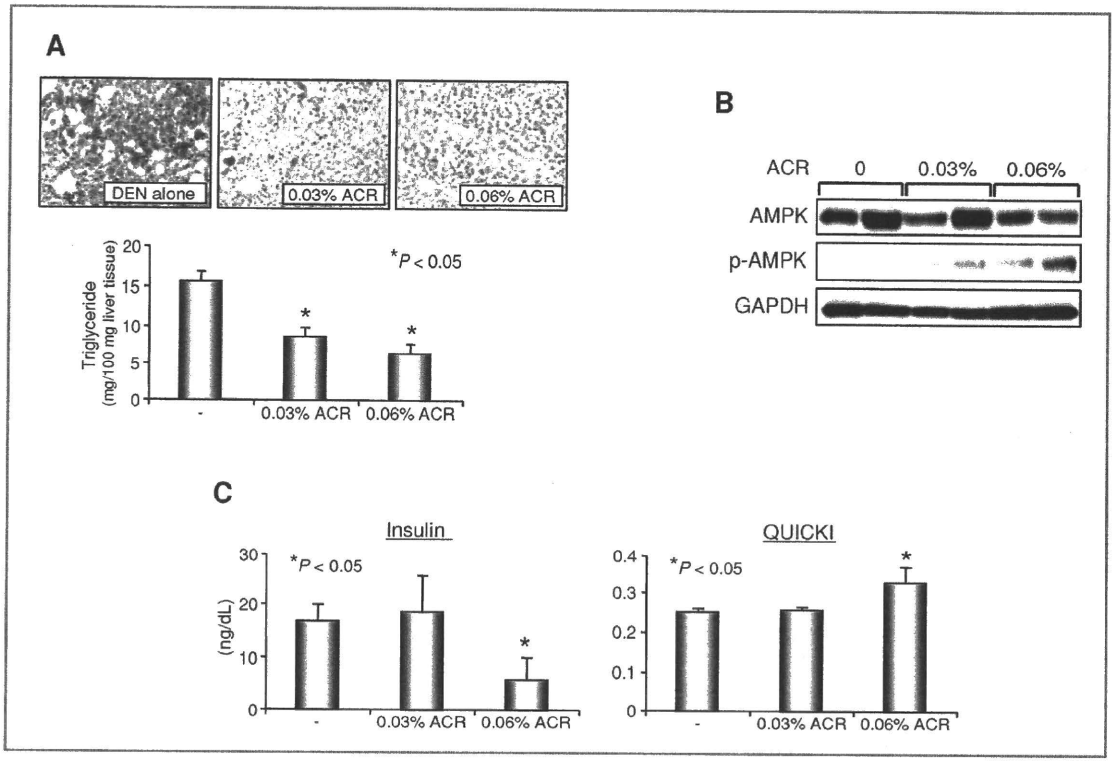


Figure 2. Effects of ACR on hepatic steatosis, the activation of the AMPK protein in the liver, and the levels of serum insulin and insulin sensitivity in DEN-treated *db/db* mice. A, frozen liver sections from DEN-exposed mice treated with or without ACR were stained with Sudan III to show steatosis (top). Hepatic lipids were extracted from the frozen livers of these mice, and the triglyceride levels were measured (bottom). B, the total proteins were extracted from the livers of DEN-treated mice, and the expression levels of the AMPK and p-AMPK proteins were examined by Western blot analysis, using the respective antibodies. A GAPDH antibody served as a loading control. C, the serum concentration of insulin was measured by enzyme immunoassay (left). The QUICKI value was calculated to evaluate insulin sensitivity (right). Values are the mean ± SD. *, $P < 0.05$ vs. ACR-untreated group.

prevents the development of obesity-related liver cell adenomas, and these effects are associated with improvement of hepatic steatosis and insulin resistance. Therefore, the findings of the present study, together with the results of previous studies using BCAA (3, 4), suggest that improvement of metabolic abnormalities by pharmaceutical or nutritional intervention might be an effective strategy for inhibiting obesity-related liver tumorigenesis.

Several biological effects of ACR are relevant to the prevention of obesity-related hepatotumorigenesis. First, it should be noted that ACR inhibits RXRα phosphorylation by suppressing the Ras/ERK signaling pathway in the livers of DEN-treated *db/db* mice. These findings are consistent with those of previous *in vitro* studies (15, 23, 24), but this is the first *in vivo* experiment, and the results seem to be significant because RXRα malfunction due to the phosphorylation by Ras-ERK plays a role in liver carcinogenesis and phosphorylated RXRα is therefore a critical target for HCC chemoprevention (10, 21). ACR suppresses the growth of HCC cells by inhibiting RXRα phosphorylation and restoring its original function as a master regulator of nuclear receptors (15, 22–24). Therefore, the expression levels of the *RARβ*, *$p21^{CIP1}$*, *cyclin D1*, *c-Fos*, and *c-Jun* genes, which are ACR targets (12–15, 28), were notably regulated by treatment with this agent. Among these molecules, RARβ seems to be the most important with respect to the induction of apoptosis (36). The upregulation of $p21^{CIP1}$, which negatively modulates cell-cycle progression, also activates the promoter region of the *RARβ* gene (37). Because RARβ can form a heterodimer with RXRα and thus synergistically inhibit the growth of HCC cells (14, 15), its induction might also have played a role in preventing the development of liver tumors in the present study. In addition, $p21^{CIP1}$ induction, which might be caused by activation of transforming growth factor (TGF)-β, also contributes to prevent the development of liver neoplasms because TGF-β induces senescence and inhibits growth in HCC cells by upregulating $p21^{CIP1}$ and ACR can activate latent TGF-β in liver stellate cells (38, 39).

Next, the effects of ACR in improving hepatic steatosis and insulin resistance, both of which accelerate HCC development (7–9), are discussed. These effects might also

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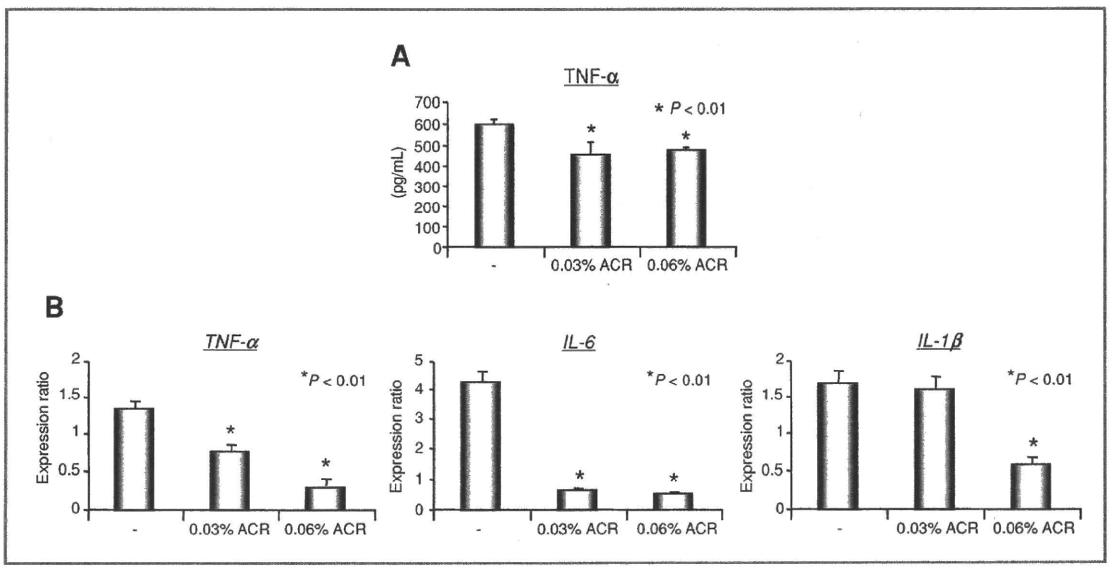


Figure 3. Effects of ACR on the serum levels of TNF-α and the expression levels of *TNF-α*, *IL-6*, and *IL-1β* mRNA in the livers of DEN-treated *db/db* mice. A, the serum concentration of TNF-α was measured by enzyme immunoassay. B, the expression levels of *TNF-α*, *IL-6*, and *IL-1β* mRNA were examined by quantitative real-time RT-PCR using specific primers. The expression levels of these mRNAs were normalized to the level of the β-*actin* mRNA. Values are the mean ± SD. *, $P < 0.01$ vs. ACR-untreated group.

be associated with RXRα dephosphorylation, as RXR can control insulin sensitization and lipid metabolism by forming a heterodimer with peroxisome proliferator-activated receptor (PPAR), an important molecule in the regulation of lipid homeostasis and energy metabolism (40). This speculation is interesting because the inhibition of RXRα phosphorylation and the activation of the RXR/PPAR heterodimer are also activities that cooperatively inhibit the growth of cancer cells (41). In addition, ACR might improve these metabolic abnormalities by activating AMPK, which increases glucose uptake and fatty acid oxidation but decreases fatty acid synthesis (33). This is another positive finding with regard to the prevention of hepatotumorigenesis because decreased AMPK activation is implicated in tumor development and therefore may be a promising target for cancer chemoprevention (42, 43). For instance, a human study suggests that metformin, an AMPK activator used to treat type 2 diabetes mellitus, reduces the cancer risk in diabetic patients (44). Dietary energy restriction suppresses mammary tumorigenesis in rats by increasing the levels of activated AMPK (45). Pitavastatin, a lipophilic statin, was found to prevent obesity- and diabetes-related colon carcinogenesis in mice by activating AMPK in the colonic mucosa (29). These reports suggest the possibility that activation of AMPK by ACR aided in suppressing the development of obesity-related liver cells adenomas, as observed in the present study.

Insulin resistance and lipid accumulation in the liver produce inflammatory changes in the liver (7–9). ACR might decrease the serum levels of TNF-α and the expression levels of *TNF-α*, *IL-6*, and *IL-1β* mRNA in the livers of experimental mice by improving hepatic steatosis and insulin resistance. These findings are significant because obesity-related HCC development clearly depends on enhanced production of TNF-α and IL-6, which cause hepatic inflammation and activate ERK and Stat3 (34). TNF-α, which lies at the core of the association between obesity and insulin resistance (46), contributes to obesity-induced IL-6 production and hepatocarcinogenesis (34). IL-6 is a major Stat3 activator in the liver, and the activation of the IL-6–Stat3 axis plays a critical role in HCC development (47, 48). In addition, uncontrolled activation of the Ras/ERK and Jak/Stat pathways is essential for HCC development (49). In the present study, ubiquitous activation of Ras-ERK signaling presumably caused accumulation of the p-RXRα protein in the liver of the obese mice. Our findings indicate that the effects of ACR in improving the inflammatory response and inhibiting Ras-ERK and Stat3 activation are crucial to prevent the development of obesity-related liver tumors.

Finally, it should be emphasized again that prevention of HCC by targeting hepatic steatosis, insulin resistance, and the state of chronic inflammation, which are caused by dysregulation of energy homeostasis, might be one of the promising strategies for the treatment of obese individuals who are at an increased risk of developing HCC (3, 4). ACR seems to be potentially effective and critical candidate for this purpose because it can improve hepatic steatosis and insulin resistance while also attenuating chronic inflammation. It inhibits RXRα phosphorylation induced by

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Ras-ERK activation, which might be associated with excess adipose tissue, and this effect is also important for preventing obesity-related liver tumorigenesis. The findings of the present study, together with the results of previous clinical trials indicating that ACR can significantly prevent the development of HCC in patients with viral cirrhosis without causing serious adverse effects (17–19), encourage the clinical usage of this agent for cirrhotic patients with obesity and diabetes. On the other hand, careful observation is required to apply a retinoid in clinical practice because of its potential toxicity. For instance, ACR may worsen hypertriglyceridemia in obese and diabetic subjects, which is a side effect observed in previous clinical trial (17), limiting the application of ACR to such subjects.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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## References

1. El-Serag HB, Tran T, Everhart JE. Diabetes increases the risk of chronic liver disease and hepatocellular carcinoma. Gastroenterology 2004;126:460–8.
2. El-Serag HB, Rudolph KL. Hepatocellular carcinoma: epidemiology and molecular carcinogenesis. Gastroenterology 2007;132:2557–76.
3. Muto Y, Sato S, Watanabe A, Moriwaki H, Suzuki K, Kato A, et al. Overweight and obesity increase the risk for liver cancer in patients with liver cirrhosis and long-term oral supplementation with branched-chain amino acid granules inhibits liver carcinogenesis in heavier patients with liver cirrhosis. Hepatol Res 2006;35:204–14.
4. Iwasa J, Shimizu M, Shiraki M, Shirakami Y, Sakai H, Terakura Y, et al. Dietary supplementation with branched-chain amino acids suppresses diethylnitrosamine-induced liver tumorigenesis in obese and diabetic C57BL/KsJ-db/db mice. Cancer Sci 2010;101:460–7.
5. El-Serag HB, Richardson PA, Everhart JE. The role of diabetes in hepatocellular carcinoma: a case-control study among United States Veterans. Am J Gastroenterol 2001;96:2462–7.
6. Imai K, Takai K, Nishigaki Y, Shimizu S, Naiki T, Hayashi H, et al. 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. Hepatol Res 2010;40:376–82.
7. Siegel AB, Zhu AX. Metabolic syndrome and hepatocellular carcinoma: two growing epidemics with a potential link. Cancer 2009;115:5651–61.
8. Smedile A, Bugianesi E. Steatosis and hepatocellular carcinoma risk. Eur Rev Med Pharmacol Sci 2005;9:291–3.
9. Powell EE, Jonsson JR, Clouston AD. Steatosis: co-factor in other liver diseases. Hepatology 2005;42:5–13.
10. Shimizu M, Takai K, Moriwaki H. Strategy and mechanism for the prevention of hepatocellular carcinoma: phosphorylated retinoid X receptor alpha is a critical target for hepatocellular carcinoma chemoprevention. Cancer Sci 2009;100:369–74.
11. Muto Y, Moriwaki H. Antitumor activity of vitamin A and its derivatives. J Natl Cancer Inst 1984;73:1389–93.
12. Suzui M, Masuda M, Lim JT, Albanese C, Pestell RG, Weinstein IB. Growth inhibition of human hepatoma cells by acyclic retinoid is associated with induction of p21(CIP1) and inhibition of expression of cyclin D1. Cancer Res 2002;62:3997–4006.
13. Suzui M, Shimizu M, Masuda M, Lim JT, Yoshimi N, Weinstein IB. Acyclic retinoid activates retinoic acid receptor beta and induces transcriptional activation of p21(CIP1) in HepG2 human hepatoma cells. Mol Cancer Ther 2004;3:309–16.
14. Shimizu M, Suzui M, Deguchi A, Lim JT, Xiao D, Hayes JH, et al. Synergistic effects of acyclic retinoid and OSI-461 on growth inhibition and gene expression in human hepatoma cells. Clin Cancer Res 2004;10:6710–21.
15. Tatebe H, Shimizu M, Shirakami Y, Sakai H, Yasuda Y, Tsurumi H, et al. Acyclic retinoid synergises with valproic acid to inhibit growth in human hepatocellular carcinoma cells. Cancer Lett 2009;285:210–7.
16. Araki H, Shidoji Y, Yamada Y, Moriwaki H, Muto Y. Retinoid agonist activities of synthetic geranyl geranoic acid derivatives. Biochem Biophys Res Commun 1995;209:66–72.
17. Muto Y, Moriwaki H, Ninomiya M, Adachi S, Saito A, Takasaki KT, et al. Prevention of second primary tumors by an acyclic retinoid, polyprenoic acid, in patients with hepatocellular carcinoma. Hepatoma Prevention Study Group. N Engl J Med 1996;334:1561–7.
18. Muto Y, Moriwaki H, Saito A. Prevention of second primary tumors by an acyclic retinoid in patients with hepatocellular carcinoma. N Engl J Med 1999;340:1046–7.
19. Okita K, Matsui O, Kumada H, Tanaka K, Kaneko S, Moriwaki H, et al. Effect of peretinoin on recurrence of hepatocellular carcinoma (HCC): Results of a phase II/III randomized placebo-controlled trial. J Clin Oncol 2010;28Suppl 7s:4024.
20. Germain P, Chambon P, Eichele G, Evans RM, Lazar MA, Leid M, et al. International Union of Pharmacology. LXIII. Retinoid X receptors. Pharmacol Rev 2006;58:760–72.
21. Matsushima-Nishiwaki R, Okuno M, Adachi S, Sano T, Akita K, Moriwaki H, et al. Phosphorylation of retinoid X receptor alpha at serine 260 impairs its metabolism and function in human hepatocellular carcinoma. Cancer Res 2001;61:7675–82.
22. Yoshimura K, Muto Y, Shimizu M, Matsushima-Nishiwaki R, Okuno M, Takano Y, et al. Phosphorylated retinoid X receptor alpha loses its heterodimeric activity with retinoic acid receptor beta. Cancer Sci 2007;98:1868–74.
23. Matsushima-Nishiwaki R, Okuno M, Takano Y, Kojima S, Friedman SL, Moriwaki H. Molecular mechanism for growth suppression of human hepatocellular carcinoma cells by acyclic retinoid. Carcinogenesis 2003;24:1353–9.
24. Kanamori T, Shimizu M, Okuno M, Matsushima-Nishiwaki R, Tsurumi H, Kojima S, et al. Synergistic growth inhibition by acyclic retinoid and vitamin K2 in human hepatocellular carcinoma cells. Cancer Sci 2007;98:431–7.
25. Kagawa M, Sano T, Ishibashi N, Hashimoto M, Okuno M, Moriwaki H, et al. An acyclic retinoid, NIK-333, inhibits N-diethylnitrosamine-induced rat hepatocarcinogenesis through suppression of TGF-alpha expression and cell proliferation. Carcinogenesis 2004;25:979–85.
26. Sano T, Kagawa M, Okuno M, Ishibashi N, Hashimoto M, Yamamoto M, et al. Prevention of rat hepatocarcinogenesis by acyclic retinoid is accompanied by reduction in emergence of both TGF-alpha-expressing oval-like cells and activated hepatic stellate cells. Nutr Cancer 2005;51:197–206.
27. Frith CH, Ward JM, Turusov VS. Tumours of the liver. In: Turusor VS, Mohr U, editors. Pathology of Tumors in Laboratory Animals. Vol 2. Lyon, France: IARC Scientific Publications; 1994. p. 223–70.