Table 5 Correlation between clinical factors and d-ROM using linear regression analysis

	Pearson's correlation coefficient	*P* value
ALB (g/dL)	−0.0073	0.9621
ALT (IU/L)	−0.1007	0.5153
T-Bil (mg/dL)	−0.0044	0.9771
PLT ($\times 10^4/\mu L$)	−0.2319	0.1254
PT (%)	0.2045	0.1777
FPG (mg/dL)	0.0013	0.9935
HbA_{1c} (%)	−0.1225	0.5043
FIRI (mg/dL)	−0.0740	0.6924
HOMA-IR	−0.1590	0.3271
AFP (ng/dL)	−0.1696	0.2892
PIVKA-II (mAU/mL)	−0.0263	0.8798
Tumor size (cm)	−0.1969	0.2074

ALB albumin, *ALT* alanine aminotransferase, *T-Bil* total bilirubin, *PLT* platelets, *PT* prothrombin time, *FPG* fasting plasma glucose, *HbA_{1c}* hemoglobin A1c, *FIRI* fasting immunoreactive insulin, *HOMA-IR* Homeostatic Model Assessment of Insulin Resistance, *AFP* α-fetoprotein, *PIVKA-II* protein induced by vitamin K absence or antagonists-II

[a] HbA_{1c} is presented in National Glycohemoglobin Standardization Program units

In the present study, HCC recurrence was noted in patients with high serum d-ROM levels (≥570 Carr U, $P = 0.0036$, Fig. 1b) that reflect increased oxidative stress (Trotti et al. 2002). In particular, the 2-year recurrence rate was higher in patients with high serum d-ROM levels (Fig. 1b). We presume that this is primarily associated with the clinical characteristic mode of liver carcinogenesis, that is multicentric carcinogenesis, (occurrence) because when the whole liver was exposed to increased oxidative stress for a long duration, multiple malignant clones that can progress to HCC in the future may have been produced. In our multicentric occurrence cases ($N = 36$), an inverse correlation was actually found between d-ROM levels and recurrence-free period. In addition, intrahepatic metastasis cases showed higher d-ROM levels than non-recurrent patients. These results of the present study, together with recent reports showing the promoting effects of oxidative stress on migration, invasion, and metastasis of HCC cells (Hu et al. 2011; Chung et al. 2012), indicate that intrahepatic metastasis might also be involved, together with multicentric occurrence, in the increase in the 2-year recurrence rate. This finding suggests that increased oxidative stress is a risk factor for HCC development and that the d-ROM test could be a useful clinical diagnostic tool to predict the recurrence of HCC.

A recent clinical trial revealed that loss of the expression of CYP1A2, a major component of the hepatic cytochrome P450 oxidative system, in non-cancerous tissue is a predictive factor of recurrence after curative hepatectomy for early-stage HCC (Tanaka et al. 2011). High levels of 8-hydroxy-2′-deoxyguanosine (8-OHdG), a marker of DNA damage caused by ROS, in liver biopsy specimens is also a risk factor for HCC development in patients with chronic HCV infection (Chuma et al. 2008). These reports (Chuma et al. 2008; Tanaka et al. 2011), together with the results of the present study, strongly suggest that oxidative stress biomarkers are useful for evaluating patients at a high risk for HCC development. In particular, the results of this study are clinically relevant because the d-ROM test can be performed easily by using serum, whereas the methods used in previous studies involve the use of liver tissues obtained by invasive surgery or biopsy (Chuma et al. 2008; Tanaka et al. 2011).

Increased production of free radicals at the site of inflammation and subsequent oxidative DNA damage is a strong mechanistic link between chronic inflammation and carcinogenesis (Hussain et al. 2003). Oxidative stress is involved in chronic liver inflammation induced by viral hepatitis, alcoholic hepatitis, and non-alcoholic steatohepatitis (Day and James 1998; Loguercio and Federico 2003; Siegel and Zhu 2009). Oxidative DNA damage is enhanced in serum and liver specimens of patients with HCV infection (Sumida et al. 2000; Mahmood et al. 2004). A strong positive correlation between inflammation, intrahepatic oxidative stress, and oxidative DNA damage are also observed in the liver of HCV-associated HCC patients (Maki et al. 2007). In the present study, the median d-ROM level of all the patients with HCC was 496 Carr U (Table 1), and this value is much higher than that of healthy control individuals (250–300 Carr U) (Trotti et al. 2002). This finding may suggest that the systemic level of oxidative stress caused by liver inflammation is increased in HCC patients. The usefulness of the d-ROM test for evaluating the correlation between increased levels of systemic inflammation and oxidative stress has also been reported in previous clinical studies (Trotti et al. 2002; Hirose et al. 2009; Sugiura et al. 2011).

The present study included 3 HCV-positive patients who received interferon therapy to eliminate the virus before HCC development. Two of these patients demonstrated a sustained virological response (SVR); however, all of them showed recurrence of HCC. Three additional HCV-positive patients also received interferon therapy to prevent HCC recurrence after the initial HCC development, but none of them showed a SVR, and 2 patients suffered a relapse. The serum d-ROM levels of the enrolled patients during the follow-up period, including after curative treatment as well as at the recurrence points, were not examined in the present study. However, these measurements seem to be significant and should be performed in future studies because the levels of d-ROM might be useful as a

biomarker for assessing the effectiveness of treatment for chronic liver diseases with interferon (Morisco et al. 2004). The d-ROM test was used in a clinical trial to evaluate oxidative status as a predictive factor of the therapeutic response of interferon and ribavirin treatment in patients with chronic hepatitis C (Morisco et al. 2004). In the study, the patients with a successive long-term response had lower d-ROM levels than non-responders (Morisco et al. 2004), suggesting that the serum levels of d-ROM might help to predict long-term response to interferon/ribavirin therapy in patients with chronic viral hepatitis. Moreover, this report also suggested that antiviral therapy could possibly attenuate oxidative stress because the mean d-ROM levels were significantly decreased during the treatment (Morisco et al. 2004). Iron depletion, which can decrease the production of ROS, improves the end-of-treatment biological and histological response to interferon therapy (Fontana et al. 2000). Iron reduction also decreases the levels of 8-OHdG and risk of HCC in HCV patients (Kato et al. 2001). Future studies to determine whether targeting oxidative stress is useful for the treatment of chronic liver disease, including the prevention of HCC, and whether the d-ROM test is applicable for evaluating oxidative stress should be conducted.

Finally, in addition to the production of ROS, alteration in the antioxidant activity is also implicated in imbalance of the normal redox state and subsequent liver carcinogenesis (Sasaki 2006; Valko et al. 2007; Sakurai et al. 2008). Experimental studies have shown evidence that dietary antioxidants, for example, vitamin E, vitamin C, and selenium, play a possible role in the prevention of liver carcinogenesis (Glauert et al. 2010). Therefore, intervention trials to examine whether antioxidant supplementation decreases the serum d-ROM levels and, therefore, possibly inhibits the development of HCC should be conducted in the future. On the other hand, several clinical studies have shown that antioxidant activity is induced as an adaptive response to increased generation of ROS in patients with HCC (Clemente et al. 2007; Abel et al. 2009; Tsai et al. 2009). An increase in the activity of manganese superoxide dismutase, an antioxidant enzyme, occurs during the precancerous phase and serves as a potential biomarker for HCC (Clemente et al. 2007). Disruption of the redox balance, resulting in increased cellular antioxidant capacity, might also create an advantageous environment for the growth of HBV-associated HCC cells (Abel et al. 2009). These reports suggest that antioxidant activity could be a predictive factor for the development of HCC.

In conclusion, this is the first indication that stage I/II patients curatively treated using surgical resection or RFA who have increased serum d-ROM levels, which reflect increased oxidative stress, are liable to HCC recurrence. The d-ROM test can be used for screening patients at a high risk for HCC recurrence, and those who show increased d-ROM levels may require careful surveillance.

Acknowledgments This work was supported in part by Grants-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan (No. 22790638 to M. S. and No. 21590838 to H. M.) and by Grant-in-Aid for the 3rd Term Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health, Labour and Welfare of Japan.

Conflict of interest The authors declare no conflict of interest.

References

Abel S, De Kock M, van Schalkwyk DJ, Swanevelder S, Kew MC, Gelderblom WC (2009) Altered lipid profile, oxidative status and hepatitis B virus interactions in human hepatocellular carcinoma. Prostaglandins Leukot Essent Fat Acids 81(5–6): 391–399. doi:10.1016/j.plefa.2009.08.003

Carmeliet P (2000) Mechanisms of angiogenesis and arteriogenesis. Nat Med 6(4):389–395. doi:10.1038/74651

Chuma M, Hige S, Nakanishi M, Ogawa K, Natsuizaka M, Yamamoto Y, Asaka M (2008) 8-Hydroxy-2′-deoxy-guanosine is a risk factor for development of hepatocellular carcinoma in patients with chronic hepatitis C virus infection. J Gastroenterol Hepatol 23(9):1431–1436. doi:10.1111/j.1440-1746.2008.05502.x

Chung JS, Park S, Park SH, Park ER, Cha PH, Kim BY, Chung YM, Woo SR, Han CJ, Kim SB, Suh KS, Jang JJ, Lee K, Choi DW, Lee S, Lee GY, Hahm KB, Shin JA, Kim BS, Noh KH, Kim TW, Lee KH, Yoo YD (2012) Overexpression of Romo1 promotes production of reactive oxygen species and invasiveness of hepatic tumor cells. Gastroenterology 143(4):1084–1094 e1087. doi:10.1053/j.gastro.2012.06.038

Clemente C, Elba S, Buongiorno G, Guerra V, D'Attoma B, Orlando A, Russo F (2007) Manganese superoxide dismutase activity and incidence of hepatocellular carcinoma in patients with Child-Pugh class A liver cirrhosis: a 7-year follow-up study. Liver Int Off J Int Assoc Study Liver 27(6):791–797. doi:10.1111/j.1478-3231.2007.01485.x

Clinical Practice Guidelines for Hepatocellular Carcinoma (2010) The Japan Society of Hepatology 2009 update. Hepatol Res Off J Jpn Soc Hepatol 40(Suppl 1):2–144. doi:10.1111/j.1872-034X.2010.00650.x

Day CP, James OF (1998) Steatohepatitis: a tale of two "hits"? Gastroenterology 114(4):842–845

Dreher D, Junod AF (1996) Role of oxygen free radicals in cancer development. Eur J Cancer 32A(1):30–38

El-Serag HB (2002) Hepatocellular carcinoma: an epidemiologic view. J Clin Gastroenterol 35(5 Suppl 2):S72–S78

Fontana RJ, Israel J, LeClair P, Banner BF, Tortorelli K, Grace N, Levine RA, Fiarman G, Thiim M, Tavill AS, Bonkovsky HL (2000) Iron reduction before and during interferon therapy of chronic hepatitis C: results of a multicenter, randomized, controlled trial. Hepatology 31(3):730–736. doi:10.1002/hep.510310325

Glauert HP, Calfee-Mason K, Stemm DN, Tharappel JC, Spear BT (2010) Dietary antioxidants in the prevention of hepatocarcinogenesis: a review. Mol Nutr Food Res 54(7):875–896. doi:10.1002/mnfr.200900482

Hirose H, Kawabe H, Komiya N, Saito I (2009) Relations between serum reactive oxygen metabolites (ROMs) and various inflammatory and metabolic parameters in a Japanese population. J Atheroscler Thromb 16(2):77–82

Hu CT, Wu JR, Cheng CC, Wang S, Wang HT, Lee MC, Wang LJ, Pan SM, Chang TY, Wu WS (2011) Reactive oxygen species-mediated PKC and integrin signaling promotes tumor progression of human hepatoma HepG2. Clin Exp Metastasis 28(8):851–863. doi:10.1007/s10585-011-9416-6

Hussain SP, Hofseth LJ, Harris CC (2003) Radical causes of cancer. Nat Rev Cancer 3(4):276–285. doi:10.1038/nrc1046

Ikeda K, Saitoh S, Tsubota A, Arase Y, Chayama K, Kumada H, Watanabe G, Tsurumaru M (1993) Risk factors for tumor recurrence and prognosis after curative resection of hepatocellular carcinoma. Cancer 71(1):19–25

Imai K, Takai K, Nishigaki Y, Shimizu S, Naiki T, Hayashi H, Uematsu T, Sugihara J, Tomita E, Shimizu M, Nagaki M, Moriwaki H (2010) 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 Off J Jpn Soc Hepatol 40(4):376–382. doi:10.1111/j.1872-034X.2009.00616.x

Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D (2011) Global cancer statistics. CA Cancer J Clin 61(2):69–90. doi:10.3322/caac.20107

Kato J, Kobune M, Nakamura T, Kuroiwa G, Takada K, Takimoto R, Sato Y, Fujikawa K, Takahashi M, Takayama T, Ikeda T, Niitsu Y (2001) Normalization of elevated hepatic 8-hydroxy-2′-deoxyguanosine levels in chronic hepatitis C patients by phlebotomy and low iron diet. Cancer Res 61(24):8697–8702

Koike Y, Shiratori Y, Sato S, Obi S, Teratani T, Imamura M, Hamamura K, Imai Y, Yoshida H, Shiina S, Omata M (2000) Risk factors for recurring hepatocellular carcinoma differ according to infected hepatitis virus-an analysis of 236 consecutive patients with a single lesion. Hepatology 32(6):1216–1223. doi:10.1053/jhep.2000.20237

Kudo M, Kubo S, Takayasu K, Sakamoto M, Tanaka M, Ikai I, Furuse J, Nakamura K, Makuuchi M (2010) Response evaluation criteria in cancer of the liver (RECICL) proposed by the Liver Cancer Study Group of Japan (2009 revised version). Hepatol Res Off J Jpn Soc Hepatol 40(7):686–692. doi:10.1111/j.1872-034X.2010.00674.x

Liver Cancer Study Group of Japan (2010) General rules for the clinical and pathological study of primary liver cancer, 3rd English edn. Kanehara & Co., Ltd., Tokyo

Loguercio C, Federico A (2003) Oxidative stress in viral and alcoholic hepatitis. Free Radic Biol Med 34(1):1–10

Mahmood S, Kawanaka M, Kamei A, Izumi A, Nakata K, Niiyama G, Ikeda H, Hanano S, Suehiro M, Togawa K, Yamada G (2004) Immunohistochemical evaluation of oxidative stress markers in chronic hepatitis C. Antioxid Redox Signal 6(1):19–24. doi:10.1089/152308604771978318

Maki A, Kono H, Gupta M, Asakawa M, Suzuki T, Matsuda M, Fujii H, Rusyn I (2007) Predictive power of biomarkers of oxidative stress and inflammation in patients with hepatitis C virus-associated hepatocellular carcinoma. Ann Surg Oncol 14(3):1182–1190. doi:10.1245/s10434-006-9049-1

Morisco F, Verde V, Fogliano V, Ritieni A, Marmo R, De Luise G, Tuccillo C, Caporaso N (2004) Oxidative status in chronic hepatitis C: the influence of antiviral therapy and prognostic value of serum hydroperoxide assay. Free Radic Res 38(6):573–580

Moriya K, Nakagawa K, Santa T, Shintani Y, Fujie H, Miyoshi H, Tsutsumi T, Miyazawa T, Ishibashi K, Horie T, Imai K, Todoroki T, Kimura S, Koike K (2001) Oxidative stress in the absence of inflammation in a mouse model for hepatitis C virus-associated hepatocarcinogenesis. Cancer Res 61(11):4365–4370

Sakurai T, He G, Matsuzawa A, Yu GY, Maeda S, Hardiman G, Karin M (2008) Hepatocyte necrosis induced by oxidative stress and IL-1 alpha release mediate carcinogen-induced compensatory proliferation and liver tumorigenesis. Cancer Cell 14(2):156–165. doi:10.1016/j.ccr.2008.06.016

Sasaki Y (2006) Does oxidative stress participate in the development of hepatocellular carcinoma? J Gastroenterol 41(12):1135–1148. doi:10.1007/s00535-006-1982-z

Seronello S, Sheikh MY, Choi J (2007) Redox regulation of hepatitis C in nonalcoholic and alcoholic liver. Free Radic Biol Med 43(6):869–882. doi:10.1016/j.freeradbiomed.2007.05.036

Siegel AB, Zhu AX (2009) Metabolic syndrome and hepatocellular carcinoma: two growing epidemics with a potential link. Cancer 115(24):5651–5661. doi:10.1002/cncr.24687

Sugiura T, Dohi Y, Takase H, Yamashita S, Tanaka S, Kimura G (2011) Increased reactive oxygen metabolites is associated with cardiovascular risk factors and vascular endothelial damage in middle-aged Japanese subjects. Vasc Health Risk Manag 7:475–482. doi:10.2147/VHRM.S23097

Sumida Y, Nakashima T, Yoh T, Nakajima Y, Ishikawa H, Mitsuyoshi H, Sakamoto Y, Okanoue T, Kashima K, Nakamura H, Yodoi J (2000) Serum thioredoxin levels as an indicator of oxidative stress in patients with hepatitis C virus infection. J Hepatol 33(4):616–622

Tanaka S, Mogushi K, Yasen M, Ban D, Noguchi N, Irie T, Kudo A, Nakamura N, Tanaka H, Yamamoto M, Kokudo N, Takayama T, Kawasaki S, Sakamoto M, Arii S (2011) Oxidative stress pathways in noncancerous human liver tissue to predict hepatocellular carcinoma recurrence: a prospective, multicenter study. Hepatology 54(4):1273–1281. doi:10.1002/hep.24536

Toyama T, Hiramatsu N, Yakushijin T, Oze T, Nakanishi F, Yasumaru M, Mochizuki K, Kanto T, Takehara T, Kasahara A, Hayashi N (2008) A new prognostic system for hepatocellular carcinoma including recurrent cases: a study of 861 patients in a single institution. J Clin Gastroenterol 42(3):317–322. doi:10.1097/MCG.0b013e3180ebe790

Trotti R, Carratelli M, Barbieri M (2002) Performance and clinical application of a new, fast method for the detection of hydroperoxides in serum. Panminerva Med 44(1):37–40

Tsai SM, Lin SK, Lee KT, Hsiao JK, Huang JC, Wu SH, Ma H, Wu SH, Tsai LY (2009) Evaluation of redox statuses in patients with hepatitis B virus-associated hepatocellular carcinoma. Ann Clin Biochem 46(Pt 5):394–400. doi:10.1258/acb.2009.009029

Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, Telser J (2007) Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem Cell Biol 39(1):44–84. doi:10.1016/j.biocel.2006.07.001

Vassalle C, Pratali L, Boni C, Mercuri A, Ndreu R (2008) An oxidative stress score as a combined measure of the pro-oxidant and anti-oxidant counterparts in patients with coronary artery disease. Clin Biochem 41(14–15):1162–1167. doi:10.1016/j.clinbiochem.2008.07.005

Watanabe N, Takai K, Imai K, Shimizu M, Naiki T, Nagaki M, Moriwaki H (2011) Increased levels of serum leptin are a risk factor for the recurrence of stage I/II hepatocellular carcinoma after curative treatment. J Clin Biochem Nutr 49(3):153–158. doi:10.3164/jcbn.10-149

Hepatology Research 2013; 43: 106–112 doi: 10.1111/hepr.12004

Special Report

Nutritional status and quality of life in current patients with liver cirrhosis as assessed in 2007–2011

Makoto Shiraki,[1] Shuhei Nishiguchi,[2] Masaki Saito,[2] Yoshitaka Fukuzawa,[3] Toshihiko Mizuta,[4] Masaki Kaibori,[5] Tatsunori Hanai,[1] Kayoko Nishimura,[6] Masahito Shimizu,[1] Hisashi Tsurumi[1] and Hisataka Moriwaki[1]

[1]*The First Department of Internal Medicine, Gifu University School of Medicine, Gifu,* [2]*Division of Hepatobiliary and Pancreatic Disease, Department of Internal Medicine, Hyogo College of Medicine, Hyogo,* [3]*Medical Education Center, Aichi Medical University School of Medicine, Aichi,* [4]*Department of Internal Medicine, Saga Medical School, Saga University, Saga,* [5]*Department of Surgery, Kansai Medical University, Osaka, and* [6]*Center for Nutrition Support and Infection Control, Gifu University Hospital, Gifu, Japan*

Aim: Current guidelines recommended adequate nutritional support for patients with liver cirrhosis to improve clinical outcome and quality of life (QOL). However, these evidences were obtained more than 10 years ago when malnutrition prevailed. In recent years, the impact of obesity on liver damage and carcinogenesis has grown. We attempted to elucidate the nutritional state and QOL in present cirrhotics.

Methods: A research group supported by the Ministry of Health, Labor and Welfare of Japan recruited 294 cirrhotics between 2007 and 2011. Subjects comprised 171 males and 123 females, 158 of whom had hepatocellular carcinoma (HCC) and Child–Pugh grades A : B : C were 154:91:49. Anthropometry, blood biochemistry and indirect calorimetry were conducted, and QOL was measured using Short Form-8.

Results: The mean body mass index (BMI) of all patients was 23.1 ± 3.4 kg/m^2, and 31% showed obesity (BMI ≥ 25.0). In subjects without ascites, edema or HCC, mean BMI was 23.6 ± 3.6, and 34% had obesity. Protein malnutrition defined as serum albumin of less than 3.5 g/dL and energy malnutrition as respiratory quotient of less than 0.85 appeared in 61% and 43%, respectively, and protein-energy malnutrition (PEM) in 27% of all subjects. Among subjects without HCC, each proportion was 67%, 48% and 30%, respectively. QOL was significantly lower on all subscales than Japanese national standard values, but was similar regardless the presence or absence of HCC.

Conclusion: While PEM is still present in liver cirrhosis, an equal proportion has obesity in recent patients. Thus, in addition to guidelines for PEM, establishment of nutrition and exercise guidelines seems essential for obese patients with liver cirrhosis.

Key words: body mass index, energy malnutrition, liver cirrhosis, protein malnutrition, quality of life

INTRODUCTION

BECAUSE THE LIVER plays the central role in nutrient and fuel metabolism, protein-energy malnutrition (PEM) is common in patients with liver cirrhosis.[1,2] Moreover, such malnutrition leads to poor prognosis and decline in the quality of life (QOL) of cirrhotics.[3,4]

Branched-chain amino acid (BCAA) administration for protein malnutrition raises the serum albumin level and improves the QOL and survival of patients with liver cirrhosis.[5–8] Treatment with late-evening snack (LES) for energy malnutrition improves respiratory quotient (RQ), liver dysfunction and QOL.[9,10]

Therefore, the guidelines for the treatment of liver cirrhosis by Japanese Society of Gastroenterology,[11] American Society for Parenteral and Enteral Nutrition[12] and European Society for Clinical Nutrition and Metabolism[13] recommend such nutritional therapy.

However, these evidences were obtained in the cirrhotic patients recruited from 1995–2000, where protein or energy malnutrition prevailed in 50–87%.[1–4] In contrast, in the next 10 years, obesity rate in the cirrhotic patients rose to approximately 30%.[14] More recently, non-alcoholic steatohepatitis (NASH) or the

Correspondence: Dr Makoto Shiraki, The First Department of Internal Medicine, Gifu University School of Medicine, 1-1 Yanagido, Gifu 501-1194, Japan. Email: mshiraki-gif@umin.ac.jp
Received 11 August 2012; revision 7 October 2012; accepted 15 October 2012.

hepatic inflammation, fibrosis and carcinogenesis due to obesity became the topics.[14–16]

Therefore, it is essential to re-evaluate a nourishment state of the current cirrhotic patients to update the guidelines. In this report, we investigated comprehensive data on the nourishment state and QOL in a large group of patients with liver cirrhosis recruited in the years 2007–2011.

METHODS

Patients

TWO HUNDRED AND ninety-four patients with liver cirrhosis (171 men and 123 women; mean age, 68 ± 10 years) undergoing treatment between 2007 and 2011 were recruited by a Research Group (Gifu University, Hyogo College of Medicine, Aichi Medical University and Saga University) supported by the Ministry of Health, Labor and Welfare of Japan. Liver cirrhosis was diagnosed by clinical and laboratory profiles and by histological examination of liver biopsy specimens. The etiology of cirrhosis was hepatitis B virus in 35 patients, hepatitis C virus in 204, alcohol in 25, NASH in six and others in 24. Child–Pugh classification of the disease severity[17] was A in 154 cases, B in 91 cases and C in 49 cases. One hundred and fifty-eight patients had hepatocellular carcinoma (HCC), and their clinical stage was I in 41 patients, II in 41, III in 54 and IV in 22. Clinical profiles of the patients are presented in Table 1. The proportion of patients supplemented with BCAA or LES rose in parallel with the increasing grade of Child–Pugh classification. Patients with fever, HIV infection, overt infectious disease (septicemia, pneumonia, urinary tract infection), renal insufficiency or under immunomodulatory therapy were excluded. The study protocol was approved by the Medical Ethics Committee of Gifu University Graduate School of Medicine, and informed consent was obtained from all patients. The study protocol was in agreement with the 1975 Declaration of Helsinki as revised in 1983.

Hematological examinations

Blood was drawn for routine laboratory examinations in the early morning after overnight fasting on the day of metabolic studies. Serum albumin, total bilirubin, alt alanine aminotransferase, prothrombin activity and urinary nitrogen (UN) were measured with a standard clinical analyzer at the central laboratory in each hospital.

Nutritional assessment

Metabolic studies were carried out using an indirect calorimeter (Aeromonitor AE-300S; Minato Medical Science, Osaka, Japan) to estimate non-protein re-

Table 1 Clinical and biochemical profiles of patients with liver cirrhosis

	Cirrhosis ($n = 294$)	Child A ($n = 154$)	Child B ($n = 91$)	Child C ($n = 49$)	*P*
Age (years)	68 ± 10	68 ± 10	68 ± 10	68 ± 12	n.s.
Sex (male/female)	171/123	90/64	51/40	30/19	n.s.
Height (cm)	159 ± 9.1	159 ± 9.0	159 ± 9.1	159 ± 9.7	n.s.
Weight (kg)	59 ± 11	58 ± 9.6	59 ± 11	60 ± 13	n.s.
Body mass index (kg/m^2)	23.1 ± 3.4	22.9 ± 3.0	23.4 ± 3.6	23.6 ± 4.0	n.s.
Etiology (HBV/HCV/alcohol/others)	35/204/25/30	20/108/11/15	11/62/8/10	4/34/6/5	n.s.
Hepatocellular carcinoma (+/−)*	158/136	84/69	54/38	20/29	n.s.
Number of patients					
Treated with BCAA	97	35	45	17	<0.01
Supplied with LES	36	8	19	9	<0.01
Albumin (g/dL)	3.3 ± 0.6	3.6 ± 0.5	3.0 ± 0.4	2.6 ± 0.4	<0.01
Total bilirubin (mg/dL)	1.4 ± 1.8	0.9 ± 0.4	1.5 ± 1.2	3.2 ± 3.8	<0.01
Alanine aminotransferase (IU/L)	44 ± 31	43 ± 30	44 ± 29	45 ± 40	n.s.
Prothrombin time (%)	81 ± 30	91 ± 32	75 ± 23	66 ± 22	<0.01

HBV, hepatitis B virus; HCV, hepatitis C virus; BCAA, branched-chain amino acids; LES, late-evening snack; n.s., not significant.
Data are presented as number of patients or mean ± standard deviation.
Statistical analysis was performed by one-way ANOVA or contingency table analysis for distribution among Child–Pugh grades A, B and C.
*Clinical stage of hepatocellular carcinoma was I in 41 patients, II in 41, III in 54 and IV in 22.

spiratory quotient (npRQ) from measured oxygen consumption/min (VO_2), carbon dioxide production/min (VCO_2) and total urinary nitrogen using the following equation:[18–20]

$$npRQ = (1.44Vco_2 - 4.890UN)/(1.44Vo_2 - 6.04UN).$$

Measurements were performed between 07.00 and 09.00 hours while the patients were still lying in bed. The last meal was served at 18.00 hours on the previous day.

We measured height and bodyweight, and calculated body mass index (BMI).

QOL questionnaire

Health-related QOL was measured using the Short Form-8 (SF-8) questionnaire.[21–23] The SF-8 contains eight questions that provide a quantitative evaluation on each of eight subscales: (i) physical functioning (PF); (ii) role physical (RP); (iii) bodily pain (BP); (iv) general health perception (GH); (v) vitality (VT); (vi) social functioning (SF); (vii) role emotional (RE); and (viii) mental health (MH).

Statistical analysis

Data were expressed as the mean and standard deviation. Comparisons of measured values among Child–Pugh classification grade A, B and C were performed using one-way ANOVA. Comparisons of sex, etiology and the presence of HCC among Child–Pugh classification grades were performed using contingency table analysis. Measured QOL was analyzed by *z*-test or Student's *t*-test between each group. Data analysis was performed using JMP ver. 5.1J (SAS Institute Japan, Tokyo, Japan) and $P < 0.05$ was considered statistically significant.

RESULTS

BMI of the patients with liver cirrhosis

THE MEAN BMI of all patients with liver cirrhosis was 23.1 ± 3.4 kg/m^2.

The ratio of obese subjects with BMI of 25 or higher was 30.6% and that of less than 18.5 kg/m^2 was 5.1%, respectively (Fig. 1).

We then excluded patients with ascites, edema or HCC to match the present cohort with those reported in 2002.[4] The number of patents in this cohort was 95, and Child–Pugh grades A, B and C were 71:22:2, respectively. Mean BMI was 23.6 ± 3.6 kg/m^2, and BMI of less than 18.5 kg/m^2 and 25.0 kg/m^2 or higher were observed in 9.2% and 33.7%, respectively (Fig. 2).

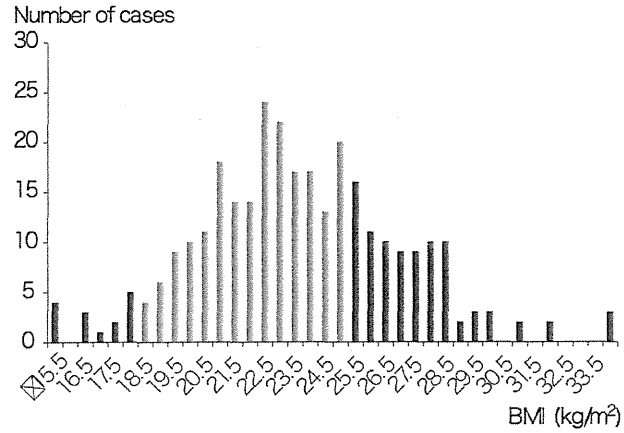


Figure 1 Distribution of body mass index (BMI) in patients with liver cirrhosis. Total number of patients = 294. Obese subjects (BMI ≥ 25) were present in 30.6%, lean ones (18.5 ≤ BMI < 25) were in 64.3% and emaciation (BMI < 18.5) was observed in 5.1%.

Incidence of protein malnutrition, energy malnutrition and PEM in patients with liver cirrhosis

We examined nutritional status in 181 patients with liver cirrhosis that underwent indirect calorimetry. In these patients, the male : female ratio was 112:69, HCC

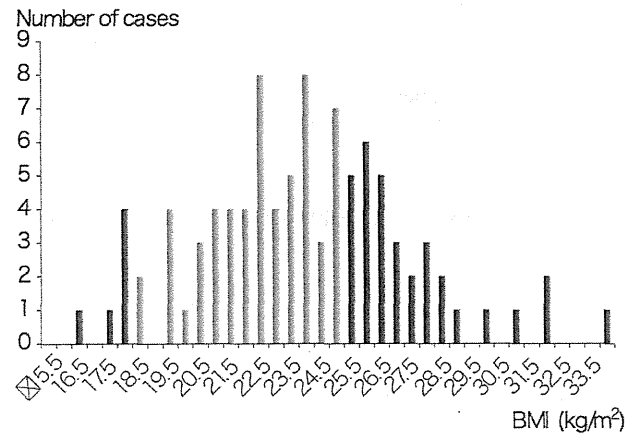


Figure 2 Distribution of body mass index (BMI) in cirrhotic patients without ascites, edema or hepatocellular carcinoma. Total number of patients = 95. Obese subjects (BMI ≥ 25) were present in 33.7%, lean ones (18.5 ≤ BMI < 25) were in 57.1% and emaciation (BMI < 18.5) was observed in 9.2%.

Table 2 Incidence of protein and energy malnutrition in patients with liver cirrhosis

Energy nutritional state	Protein nutritional state	
	Normal (%)	Malnourished (%)
Normal (%)	42 (23%)	62 (34%)
Malnourished (%)	28 (16%)	49 (27%)

Protein malnutrition was defined as serum albumin level of <3.5 g/dL and energy malnutrition as a respiratory quotient of <0.85.
Total number of patients = 181.
Data are presented as number of patients (%).

was present in 94, and Child–Pugh grades A : B : C were 90:58:33. When protein malnutrition was defined as serum albumin level of less than 3.5 g/dL and energy malnutrition as a non-protein respiratory quotient of less than 0.85, protein malnutrition was found in 61%, energy malnutrition in 43% and PEM in 27% (Table 2). Similarly, among 87 patients without HCC (Child–Pugh grades A : B : C, 36:27:24), 67% had protein malnutrition, 48% had energy malnutrition and 30% had PEM (Table 3).

Table 3 Incidence of protein and energy malnutrition in cirrhotic patients without hepatocellular carcinoma

Energy nutritional state	Protein nutritional state	
	Normal (%)	Malnourished (%)
Normal (%)	13 (15%)	32 (37%)
Malnourished (%)	16 (18%)	26 (30%)

Protein malnutrition was defined as serum albumin level of <3.5 g/dL and energy malnutrition as a respiratory quotient of <0.85.
Total number of patients = 87.
Data are presented as number of patients (%).

Table 5 Comparison of health-related quality of life in cirrhotics by the presence or absence of hepatocellular carcinoma

Subscales	Absence of hepatocellular carcinoma	Presence of hepatocellular carcinoma	*P*
Physical functioning	43.4 ± 4.9	44.2 ± 5.5	n.s.
Role physical	41.1 ± 6.3	42.1 ± 6.8	n.s.
Bodily pain	47.8 ± 5.3	48.7 ± 5.1	n.s.
General health perception	44.9 ± 4.5	45.4 ± 3.9	n.s.
Vitality	46.5 ± 4.3	48.4 ± 4.2	n.s.
Social functioning	45.3 ± 5.0	46.8 ± 5.4	n.s.
Role emotional	45.3 ± 5.0	45.8 ± 6.1	n.s.
Mental health	46.6 ± 3.9	48.5 ± 4.0	n.s.

n.s., not significant.
Data are presented as mean ± standard deviation.
Statistical analysis was performed by *z*-test between the presence and absence of hepatocellular carcinoma.

Health-related QOL of the patients with liver cirrhosis

We examined health-related QOL in 114 patients with liver cirrhosis (64 men and 50 women) using the SF-8. Sixty-two patients had HCC, and Child–Pugh grades A : B : C were 63:26:25.

Quality of life of all subjects was significantly lower on all subscales than Japanese national standard values (Table 4),[24] but no difference was observed between the presence and the absence of HCC (Table 5).

Table 4 Comparison of health-related quality of life between the Japanese national standard and the patients with liver cirrhosis

Subscales	Japanese national standard	Patients with liver cirrhosis	*P*
Physical functioning	50.1 ± 5.0	43.8 ± 5.2	<0.01
Role physical	50.2 ± 5.3	41.6 ± 6.6	<0.01
Bodily pain	51.3 ± 8.3	48.3 ± 5.3	<0.01
General health perception	50.6 ± 6.6	45.2 ± 4.4	<0.01
Vitality	52.4 ± 5.5	47.5 ± 4.3	<0.01
Social functioning	50.2 ± 6.6	46.1 ± 5.3	<0.01
Role emotional	51.3 ± 4.5	45.6 ± 5.7	<0.01
Mental health	53.3 ± 5.4	47.6 ± 4.0	<0.01

Data are presented as mean ± standard deviation.
Statistical analysis was performed by Student's *t*-test between the Japanese national standard[24] and the patients with liver cirrhosis.

DISCUSSION

PROTEIN-ENERGY MALNUTRITION is a common manifestation in cirrhotic patients with reported incidences as high as 50–87%.[1,2] Protein nutrition is usually evaluated by serum albumin level and, for energy nutrition, indirect calorimetry is recommended for precise analysis.[13] Energy malnutrition typically shows reduced carbohydrate oxidation, increased fat oxidation and decline in npRQ measured by indirect calorimetry. It is reported that PEM worsens prognosis and QOL in patients with liver cirrhosis.[3,4] Thus, intervention for PEM is an important issue in the clinical management of liver cirrhosis.

For this purpose, BCAA administration for protein malnutrition raises the serum albumin level and improves QOL and survival of patients with liver cirrhosis.[5–8] LES for energy malnutrition improves npRQ, liver dysfunction and QOL.[9,10] Thus, many guidelines[11–13] recommend such nutritional therapy for liver cirrhosis.

However, these evidences were obtained in the cirrhotic patients recruited from 1995 through 2000 where malnutrition prevailed but obesity was apparently less (20%)[4] than the general cohort (30%).[25] In the next 10 years, obesity rose by approximately 1.5 times in the patients with chronic liver disease in Japan.[14] In addition, presence of diabetes mellitus, hyperinsulinemia or obesity is currently regarded as a significant risk factor for liver carcinogenesis.[14–16] Furthermore, the relationship between obesity and liver inflammation and fibrosis, including NASH has become an important issue in recent years. Therefore, it is necessary to elucidate the nourishment state of the present cirrhotic patients to update guidelines. Thus, we report in this paper a comprehensive survey of the nourishment state and QOL in the present patients with liver cirrhosis.

The etiology of the 294 cirrhotics was hepatitis B virus in 11.9%, hepatitis C virus in 69.4%, alcohol in 8.5%, NASH in 2.0% and others in 8.2% in this study. In the 44th Annual Meeting of Japan Society of Hepatology in 2008 (Matsuyama), the reported etiology of 33 379 cirrhotics was hepatitis B virus in 13.9%, hepatitis C virus in 60.9%, alcohol in 13.6%, NASH in 2.1% and others in 9.5%,[26] indicating similar patient composition between two studies.

Obesity is defined by BMI of 25 or higher in Japan but by 30 or higher by World Health Organization. In this study, the mean BMI excluding patients with ascites, edema or HCC was 23.6 ± 3.6 kg/m^2 and the ratio of obese subjects with BMI of 25 or higher was 33.7% of these patients (Fig. 2). The proportion of obese people in the general population of Japan at matched age was 30.5% in 2009.[25] Thus, an equal or greater proportion of patients with liver cirrhosis has obesity than the general population of Japan at present.

The increase in obesity, or excess energy nutrition status, and subsequent impaired glucose metabolism potentially bring about an unfavorable outcome in cirrhotic patients. Actually, excess energy nutrition contributed to induce carcinogenesis in liver cirrhosis,[15,27,28] and the number of obese subjects doubled in the candidates for liver transplantation in the previous 10 years in the USA.[29–31]

As to PEM exactly defined by serum albumin and npRQ, Tajika *et al.* reported that protein malnutrition was identified in 75%, energy malnutrition in 62% and PEM in 50% of 109 patients with liver cirrhosis in 1995.[4] In our study, 87 patients without HCC composed a group to show comparable backgrounds to those by Tajika *et al.*[4] Among them, 67% had protein malnutrition, 48% had energy malnutrition and 30% had PEM (Table 3). Taken together, the protein malnutrition remains almost similar in liver cirrhosis, but the patients with energy malnutrition, particularly PEM, substantially decreased.

The above-mentioned results urge that two concerns are addressed. The first is the effect of altered nutritional state of cirrhotics on their QOL, and the second is a question if exercise should be prescribed for obese cirrhotics. Regarding QOL, reduction in bodyweight achieved by chronic liver disease patients with obesity was associated with improved liver dysfunction, histology or QOL.[32,33]

In this study, basal QOL was estimated by the SF-8, and was significantly lower on all subscales than Japanese national standard values. However, no difference was observed by the presence or absence of HCC. In contrast, QOL of cirrhotic patients significantly correlated with the grade of disease severity as defined by the Child–Pugh classification (data not shown). It was thus suggested that the degree of the hepatic functional reserve contributed to a greater extent than the progression of cancer as for QOL of cirrhotic patients.

In conclusion, while PEM is still present in liver cirrhosis, a greater proportion shows obesity in Japanese patients at present. Because exacerbated inflammation, fibrosis and carcinogenesis has been reported in obese patients with liver cirrhosis, the present findings urge revision of nutritional and, possibly, establishment of exercise guidelines for obese patients with liver cirrhosis, in addition to the current PEM guidelines.

ACKNOWLEDGMENT

THIS WORK WAS supported in part by Grants-in-Aid from the Ministry of Health, Labor, and Welfare of Japan.

REFERENCES

1 Morgan AG, McAdam WA, Walmsley GR, Horrocks JC, De Dombal FT. Nutrition in cryptogenic cirrhosis and chronic aggressive hepatitis. *Gut* 1976; **17** (2): 113–8.
2 Müller MJ. Malnutrition in cirrhosis. *J Hepatol* 1995; **23** (Suppl 1): 31–5.
3 Alberino F, Gatta A, Amodio P *et al.* Nutrition and survival in patients with liver cirrhosis. *Nutrition* 2001; **17**: 445–50.
4 Tajika M, Kato M, Mohri H *et al.* Prognostic value of energy metabolism in patients with viral liver cirrhosis. *Nutrition* 2002; **18** (3): 229–34.
5 Yoshida T, Muto Y, Moriwaki H, Yamato M. Effect of long-term oral supplementation with branched-chain amino acid granules on the prognosis of liver cirrhosis. *J Gastroenterol* 1989; **24**: 692–8.
6 Marchesini G, Bianci G, Merli M *et al.* Nutritional supplementation with branched-chain amino acids in advanced cirrhosis: a double-blind, randomized trial. *Gastroenterology* 2003; **124**: 1792–801.
7 Muto Y, Sato S, Watanabe A *et al.* Effects of oral branched-chain amino acid granules on event-free survival in patients with liver cirrhosis. *Clin Gastroenterol Hepatol* 2005; **3** (7): 705–13.
8 Fukushima H, Miwa Y, Shiraki M *et al.* Oral branched-chain amino acid supplementation improves the oxidized/reduced albumin ratio in patients with liver cirrhosis. *Hepatol Res* 2007; **37** (9): 765–70.
9 Miwa Y, Shiraki M, Kato M *et al.* Improvement of fuel metabolism by nocturnal energy supplementation in patients with liver cirrhosis. *Hepatol Res* 2000; **18** (3): 184–89.
10 Nakaya Y, Okita K, Suzuki K *et al.* BCAA-enriched snack improves nutritional state of cirrhosis. *Nutrition* 2007; **23**: 113–20.
11 The Japanese Society of Gastroenterology. *Guidelines for the Diagnosis and Treatment of Liver Cirrhosis*. Tokyo: Nankodo, 2010. (in Japanese).
12 ASPEN Board of Directors and the Clinical Guidelines Task Force. Guidelines for the use of parenteral and enteral nutrition in adult and pediatric patients. *JPEN J Parenter Enteral Nutr* 2002; **26** (Suppl 1): 73–6.
13 ESPEN. Guidelines on enteral nutrition: liver disease. *Clin Nutr* 2006; **25**: 285–94.
14 Moriwaki H, Shiraki M, Fukushima H *et al.* Long-term outcome of branched-chain amino acid treatment in patients with liver cirrhosis. *Hepatol Res* 2008; **38** (s1): S102–6.
15 Muto Y, Sato S, Watanabe A *et al.* Over weight and obesity increase the risk for HCC 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.
16 Hickman IJ, Powell EE, Prins JB *et al.* In overweight patients with chronic hepatitis C, circulating insulin is associated with hepatic fibrosis: implications for therapy. *J Hepatol* 2003; **39** (6): 1042–8.
17 Pugh RN, Murray-Lyon IM, Dawson JL, Pietroni MC, Williams R. Transection of the oesophagus for bleeding oesophageal varices. *Br J Surg* 1973; **60** (8): 646–9.
18 Kato M, Miwa Y, Tajika M, Hiraoka T, Muto Y, Moriwaki H. Preferential use of branched-chain amino acids as an energy substrate in patients with liver cirrhosis. *Intern Med* 1998; **37** (5): 429–34.
19 Müller MJ, Böttcher J, Selberg O *et al.* Hypermetabolism in clinically stable patients with liver cirrhosis. *Am J Clin Nutr* 1999; **69** (6): 1194–201.
20 Süttmann U, Ockenga J, Hoogestraat L *et al.* Resting energy expenditure and weight loss in human immunodeficiency virus-infected patients. *Metabolism* 1993; **42** (9): 1173–9.
21 Turner-Bowker DM, Bayliss MS, Ware JE, Jr Kosinski M. Usefulness of the SF-8 Health Survey for comparing the impact of migraine and other conditions. *Qual Life Res* 2003; **12**: 1003–12.
22 Ware JE, Kosinski M, Dewey JE, Gandek B. *How to Score and Interpret Single-Item Healthstatus Measures. A Manual for Users of the SF-8TM Health Survey*. Lincoln, RI: QualityMetric Inc., 2001.
23 Kuroda H, Ushio A, Miyamoto Y *et al.* Effects of branched-chain amino acid-enriched nutrient for patients with hepatocellular carcinoma following radiofrequency ablation: a one-year prospective trial. *J Gastroenterol Hepatol* 2010; **25** (9): 1550–5.
24 Fukuhara S, Suzukamo Y. *Manual of the SF-8 Japanese Version*. Kyoto: Institute for Health Outcome and Process Evaluation Research, 2004. (in Japanese).
25 Health and Welfare Statistics Association. *Journal of Health and Welfare Statistics 2010/2011*. Tokyo: Kosaido, 2011. (in Japanese).
26 Michitaka K, Nishiguchi S, Aoyagi Y *et al.* Etiology of liver cirrhosis in Japan: a nationwide survey. *J Gastroenterol* 2010; **45** (1): 86–94.
27 Yoshiji H, Noguchi R, Kitade M *et al.* Branched-chain amino acids suppress insulin-resistance-based hepatocarcinogenesis in obese diabetic rats. *J Gastroenterol* 2009; **44** (5): 483–91.
28 Iwasa J, Shimizu M, Shiraki M *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** (2): 460–7.

29 Nair S, Mason A, Eason J *et al.* Is obesity an independent risk factor for hepatocellular carcinoma in cirrhosis? *Hepatology* 2002; 36 (1): 150–5.
30 Pelletier SJ, Schaubel DE, Wei G *et al.* Effect of body mass index on the survival benefit of liver transplantation. *Liver Transpl* 2007; 13 (12): 1678–83.
31 Thuluvath PJ. Morbid obesity and gross malnutrition are both poor predictors of outcomes after liver transplantation: what can we do about it? *Liver Transpl* 2009; 15 (8): 838–41.
32 Oza N, Eguchi Y, Mizuta T *et al.* A pilot trial of body weight reduction for nonalcoholic fatty liver disease with a home-based lifestyle modification intervention delivered in collaboration with interdisciplinary medical staff. *J Gastroenterol* 2009; 44 (12): 1203–8.
33 Hickman IJ, Jonsson JR, Prins JB *et al.* Modest weight loss and physical activity in overweight patients with chronic liver disease results in sustained improvements in alanine aminotransferase, fasting insulin, and quality of life. *Gut* 2004; 53 (3): 413–9.

Int. J. Mol. Sci. **2013**, *14*, 14700-14711; doi:10.3390/ijms140714700

OPEN ACCESS

International Journal of

Molecular Sciences

ISSN 1422-0067

www.mdpi.com/journal/ijms

Article

Enhanced Development of Azoxymethane-Induced Colonic Preneoplastic Lesions in Hypertensive Rats

Takahiro Kochi [1], Masahito Shimizu [1,*], Tomohiko Ohno [1], Atsushi Baba [1], Takafumi Sumi [1], Masaya Kubota [1], Yohei Shirakami [1], Hisashi Tsurumi [1], Takuji Tanaka [2] and Hisataka Moriwaki [1]

[1] Department of Internal Medicine, Gifu University Graduate School of Medicine, Gifu 501-1194, Japan; E-Mails: kottii924@yahoo.co.jp (T.K.); tomohikooh@hotmail.com (T.O.); babagif@yahoo.co.jp (A.B.); sumieron@gmail.com (T.S.); kubota-gif@umin.ac.jp (M.K.); shirakamiyy@yahoo.co.jp (Y.S.); htsuru@gifu-u.ac.jp (H.T.); hmori@gifu-u.ac.jp (H.M.)

[2] Department of Tumor Pathology, Gifu University Graduate School of Medicine, Gifu 501-1194, Japan; E-Mail: takutt@toukaisaibou.co.jp

* Author to whom correspondence should be addressed; E-Mail: shimim-gif@umin.ac.jp; Tel.: +81-58-230-6313; Fax: +81-58-230-6310.

Received: 2 May 2013; in revised form: 25 June 2013 / Accepted: 27 June 2013 / Published: 15 July 2013

Abstract: Metabolic syndrome is associated with an increased risk of colorectal cancer. This study investigated the impact of hypertension, a component of metabolic syndrome, on azoxymethane (AOM)-induced colorectal carcinogenesis using SHRSP/Izm (SHRSP) non-diabetic/hypertensive rats and SHRSP.Z-*Lepr*^{fa}/IzmDmcr (SHRSP-ZF) diabetic/hypertensive rats. Male 6-week-old SHRSP, SHRSP-ZF, and control non-diabetic/normotensive Wister Kyoto/Izm (WKY) rats were given 2 weekly intraperitoneal injections of AOM (20 mg/kg body weight). Two weeks after the last injection of AOM, the SHRSP and SHRSP-ZF rats became hypertensive compared to the control WKY rats. Serum levels of angiotensin-II, the active product of the renin-angiotensin system, were elevated in both SHRSP and SHRSP-ZF rats, but only the SHRSP-ZF rats developed insulin resistance, dyslipidemia, and hyperleptinemia and exhibited an increase in adipose tissue. The development of AOM-induced colonic preneoplastic lesions and aberrant crypts foci, was significantly accelerated in both SHRSP and SHRSP-ZF hypertensive rats, compared to WKY normotensive rats. Furthermore, induction of oxidative stress and exacerbation of inflammation were observed in the colonic mucosa and systemically in SHRSP and SHRSP-ZF rats. Our findings suggest that hypertension plays a role in the early

stage of colorectal carcinogenesis by inducing oxidative stress and chronic inflammation, which might be associated with activation of the renin-angiotensin system.

Keywords: hypertension; colon carcinogenesis; oxidative stress; inflammation; angiotensin-II

1. Introduction

Obesity-related systemic metabolic dysfunctions such as diabetes mellitus, hypertension, and dyslipidemia are collectively known as metabolic syndrome (Mets) and pose serious health problems throughout the world [1,2]. In addition to the morbidity associated with these metabolic disorders, recent studies have revealed that Mets is linked to an increased risk of cancer in several organ sites including the colorectum [3–8]. Several pathophysiological mechanisms for this association have been described, including the emergence of insulin resistance, the state of chronic inflammation, induction of oxidative stress, and occurrence of adipokine imbalance [5,6]. In particular, diabetes is closely associated with the development of colorectal cancer (CRC) as obesity is the main determinant of insulin resistance and hyperinsulinemia [7].

Epidemiological studies have also revealed that hypertension may increase the risk of CRC [3,4]. The renin-angiotensin system is a key regulator of cardiovascular function, and its activation is involved in the etiology of Mets, especially hypertension [9]. There is increasing evidence that the renin-angiotensin system may have paracrine and autocrine functions with regard to tissue oxidative stress and chronic inflammation, as well as cellular proliferation and apoptosis [10–14]. In addition, dysregulation of the renin-angiotensin system has been reported to occur in human malignancies and has been shown to influence cancer cell migration, invasion, and metastasis, all of which are associated with a poor prognosis [10,11,14]. However, the precise mechanisms by which hypertension plays a role in the early stage of colorectal carcinogenesis remain unclear.

The stroke-prone spontaneously hypertensive rat (SHRSP) is a substrain of the spontaneously hypertensive rat (SHR), crossed and further inbred with selected offspring of parents that died of stroke. The SHRSP rats have a higher blood pressure than SHR rats and readily develop apoplexy. The crossing of SHRSP rats with Zucker Fatty (ZF) rats produces SHRSP.*Z-Lepr*^{fa}/IzmDmcr (SHRSP-ZF) rats, which develop hypertension and become obese due to the leptin receptor *OB-rb* gene mutation carried by ZF rats [15]. SHRSP-ZF rats therefore exhibit a phenotype similar to human Mets and thus may be a useful model to investigate the molecular mechanisms underlying hypertension-related metabolic abnormalities [15,16]. However, colorectal carcinogenesis models using these rats have not been established.

The objective of this study was to determine the susceptibility of SHRSP-ZF and SHRSP rats to azoxymethane (AOM)-induced colorectal carcinogenesis and the utility of these rats as models for Mets, in particular, as models for hypertension-associated colorectal carcinogenesis, that appropriately reflect the pathological conditions of human Mets.

2. Results and Discussion

2.1. General Observations

Table 1 compares the mean body weights, adipose tissue weights, and blood pressures (systolic and diastolic) at the end of the study (10 weeks of age) between 3 groups (Group 1, Wister Kyoto/Izm [WKY] rats; Group 2, SHRSP rats; and Group 3, SHRSP-ZF rats) that received AOM. The mean body weights of WKY ($p < 0.001$) and SHRSP-ZF ($p < 0.05$) rats were significantly higher than that of SHRSP rats, but there was no significant difference between the WKY and SHRSP-ZF rats. There was a significant increase in the mean adipose tissue weights in SHRSP-ZF rats compared to WKY ($p < 0.001$) and SHRSP rats ($p < 0.05$). The systolic and diastolic blood pressures of SHRSP and SHRSP-ZF rats were markedly higher than those of WKY rats ($p < 0.001$). However, compared to SHRSP-ZF rats, SHRSP rats had marked hypertension ($p < 0.05$).

Table 1. Body, liver and adipose weights, BMI and blood pressure of rats.

Group NO.	Strain	No.	Body weight (g)	Relative adipose tissue weight (g/100g body weight) [a]	Blood pressure (mmHg)	
					Systolic	Diastolic
Group 1	WKY [b]	8	256.5 ± 11.7 [e]	0.72 ± 0.16	127 ± 12.8	92 ± 4.9
Group 2	SHRSP [c]	8	218.9 ± 8.0 [f]	0.77 ± 0.16	188 ± 12.5 [f]	141 ± 10.6 [f]
Group 3	SHRSP-ZF [d]	8	270.1 ± 23.4 [g]	1.64 ± 0.17 [f,g]	169 ± 13.7 [f,g]	129 ± 9.0 [f,g]

[a] White adipose tissue of the periorchis; [b] Wister Kyoto/Izm; [c] stroke-prone spontaneously hypertensive/Izm; [d] SHRSP.Z-*Leprfa*/IzmDmcr; [e] Mean ± SD; [f] Significantly different from group 1 by Tukey-Kramer Multiple Comparison Test ($p < 0.001$); [g] Significantly different from group 2 by Tukey-Kramer Multiple Comparison Test ($p < 0.05$).

2.2. Serum Parameters of the Experimental Rats

As shown in Table 2, the serum levels of glucose and insulin significantly increased, but the value of QUICKI, a useful index of insulin sensitivity [17], was decreased in SHRSP-ZF rats compared to WKY and SHRSP rats ($p < 0.05$). The serum levels of leptin, non-esterified fatty acid (NEFA), and triglycerides in SHRSP-ZF rats were also significantly higher than those in WKY and SHRSP rats ($p < 0.05$). These findings suggest that SHRSP-ZF rats developed insulin resistance, hyperleptinemia, and dyslipidemia, all of which are frequently observed in human Mets patients. There were no significant differences in these serum components between WKY and SHRSP rats. The SHRSP and SHRSP-ZF rats did, however, have significantly elevated levels of serum angiotensin-II (AT-II), the active product of the renin-angiotensin system [18], compared to the WKY rats ($p < 0.05$), indicating that the renin-angiotensin system is activated in these hypertensive rats.

Table 2. Serum parameters of the experimental rats.

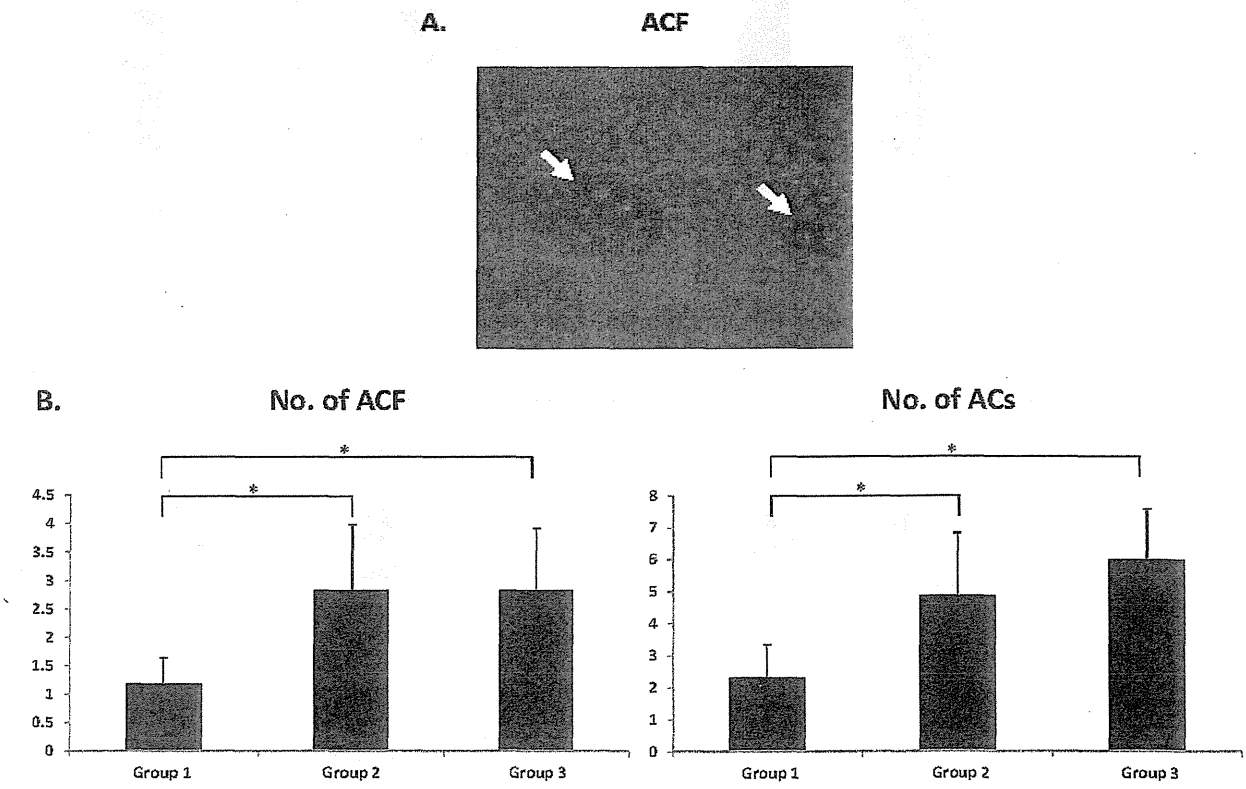
	Glucose (mg/dL)	Insulin (μIU/mL)	Quicki	Leptin (pg/mL)	NEFA (mEq/L)	Triglyceride (mg/dL)	Angiotensin II (ng/mL)
Group 1	85.4 ± 11.7	15.81 ± 0.35	0.313 ± 0.010	11.2 ± 3.6	0.459 ± 0.03	27.1 ± 7.4	352.6 ± 38.1
Group 2	83.5 ± 12.3	17.00 ± 1.39	0.320 ± 0.008	12.2 ± 3.4	0.419 ± 0.05	39.6 ± 14.1	494.4 ± 75.6 [b]
Group 3	120.0 ± 14.2 [b,c]	25.60 ± 8.98 [b,c]	0.291 ± 0.010 [b,c]	102.7 ± 30.6 [b,c]	0.538 ± 0.03 [b,c]	257.1 ± 79.4 [b,c]	500.9 ± 42.5 [b]

[a] Mean ± SD; [b] Significantly different from group 1 by Tukey-Kramer Multiple Comparison Test ($p < 0.05$); [c] Significantly different from group 2 by Tukey-Kramer Multiple Comparison Test ($p < 0.05$).

2.3. Development of Colonic Preneoplastic Lesions

Irrespective of the rat strain, aberrant crypt foci (ACF) (Figure 1A) were observed in the colon of all rats given AOM at the end of the study. However, the number and size (aberrant crypts [ACs] per cm^2) of ACF were significantly greater in both the SHRSP and SHRSP-ZF rats than in the WKY rats (Figure 1B; $p < 0.05$). There was no significant difference in the development of ACF between SHRSP and SHRSP-ZF rats, indicating that hypertension, a common pathophysiological characteristic of these rats, plays a critical role in accelerating the development of colonic preneoplastic lesions.

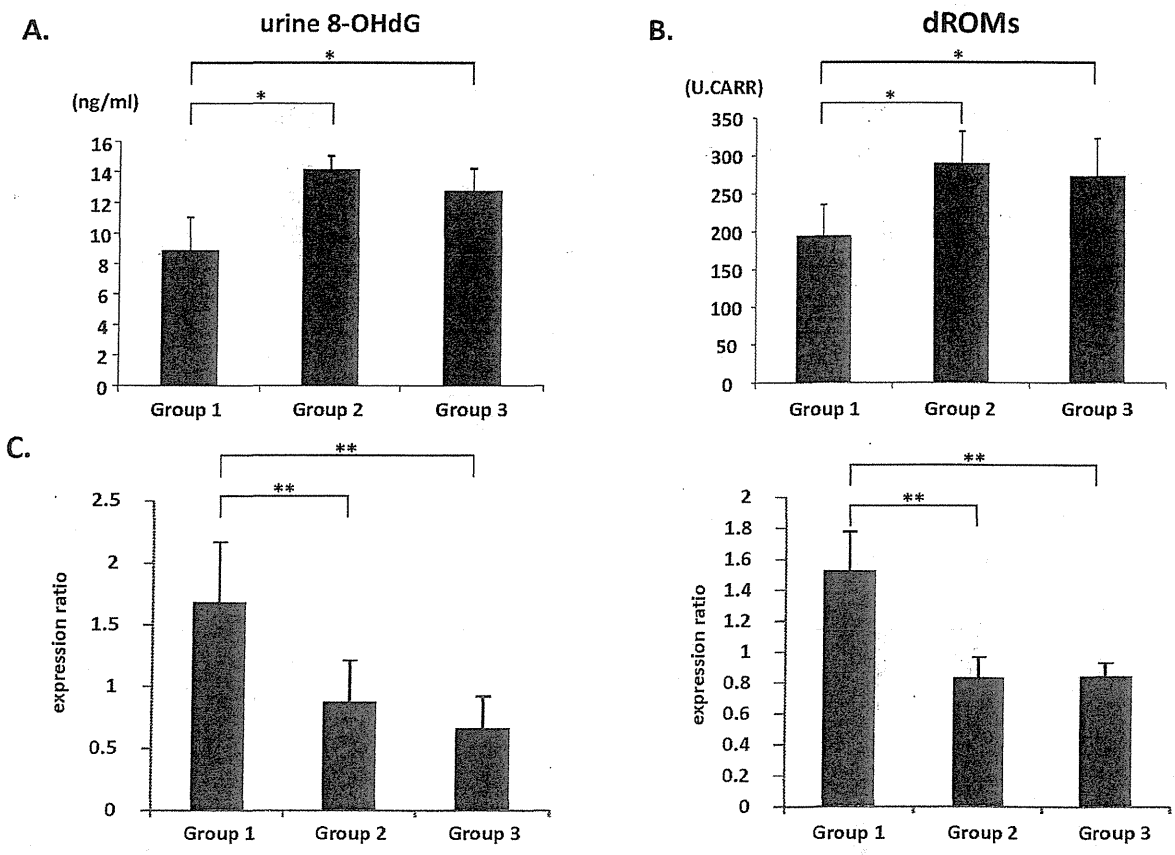
Figure 1. ACF developed in the SHRSP, SHRSP-ZF, and WKY rats that received AOM. (**A**) Representative morphology of ACF (arrows) induced by AOM stained with methylene blue in Group 2. Magnification, 40×; (**B**) Average number of ACF and ACs (/cm^2). Group 1: WKY rats, Group 2: SHRSP rats, and Group 3: SHRSP-ZF rats. The values are expressed as mean ± SD. * $p < 0.05$.



2.4. Systemic Oxidative Stress and Colonic Epithelial Expression of GPx and CAT mRNA

Oxidative stress is implicated in Mets and colorectal tumorigenesis [5]. Therefore, the levels of oxidative stress and antioxidant biomarkers in the experimental rats were assessed. Compared to the WKY rats, the SHRSP and SHRSP-ZF rats had significantly increased levels of urine 8-hydroxy-2'-deoxyguanosine (8-OHdG) (Figure 2A; $p < 0.01$), a marker of DNA damage induced by oxidative stress, and serum derivatives of reactive oxygen metabolites (d-ROM) (Figure 2B; $p < 0.01$), which reflects serum hydroperoxide levels. However, the SHRSP and SHRSP-ZF rats also had reduced expression levels of glutathione peroxidase (*GPx*) and catalase (*CAT*) mRNA, which encode antioxidant enzymes, in the colonic epithelium (Figure 2C; $p < 0.05$). These findings suggest that systemic oxidative stress is increased, whereas colonic antioxidant activity is decreased, in both SHRSP and SHRSP-ZF hypertensive rats.

Figure 2. Measures of oxidative stress and antioxidant biomarkers' expression. (**A**) Urine 8-OHdG levels were measured by enzyme immunoassay; (**B**) Hydroperoxide levels in the serum were determined by the d-ROM test; (**C**) The expression levels of *GPx* and *CAT* mRNA in the colonic epithelium were examined by quantitative real-time RT-PCR using specific primers. The values are expressed as mean ± SD. * $p < 0.01$, ** $p < 0.01$.

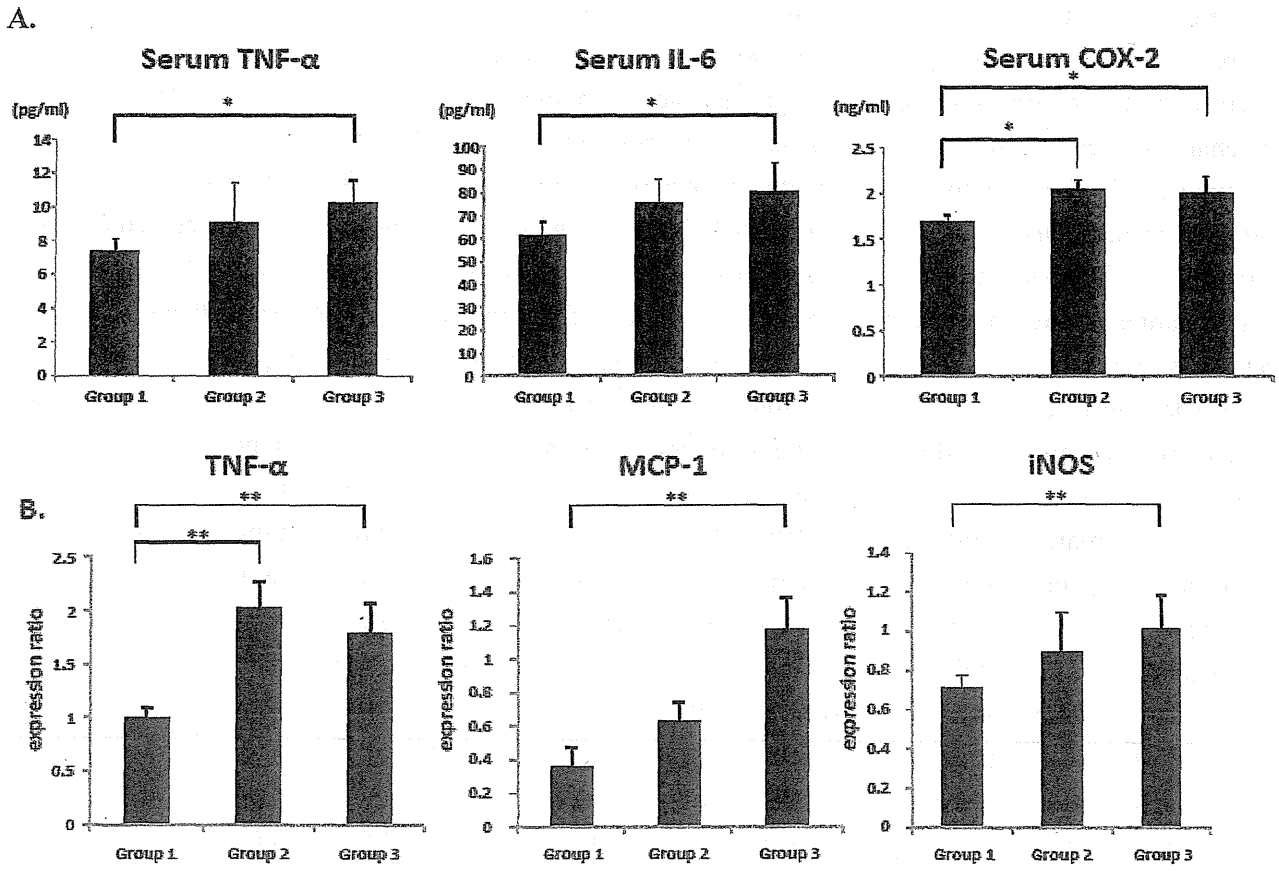


2.5. Serum and Colonic Epithelial Expression of Inflammatory Markers

Chronic inflammation plays a critical role in the pathogenesis of Mets and CRC development [5,8]. Therefore, the levels of inflammatory mediators, including tumor necrosis factor (TNF)-α, interleukin

(IL)-6, monocyte chemoattractant protein (MCP)-1, inducible nitric oxide synthase (iNOS), and cyclooxygenase (COX)-2 in hypertensive SHRSP and SHRSP-ZF rats were next examined. The serum levels of TNF-α and IL-6 in SHRSP-ZF rats were significantly elevated compared to those in WKY rats (Figure 3A; $p < 0.01$). The serum levels of COX-2 were also significantly increased in both SHRSP and SHRSP-ZF rats (Figure 3A; $p < 0.01$). In the colonic epithelium of SHRSP-ZF rats, there was a marked increase in the expression of *TNF-α*, *MCP-1*, and *iNOS* mRNA (Figure 3B; $p < 0.05$ compared to WKY rats). Compared to the WKY rats, the expression of *TNF-α* mRNA in the colonic epithelium of SHRSP rats was also significantly increased (Figure 3B; $p < 0.05$).

Figure 3. Serum levels of TNF-α, IL-6, and COX-2 and the expression levels of *TNF-α*, *MCP-1*, and *iNOS* mRNA in the colonic epithelium. (**A**) The serum concentrations of TNF-α, IL-6, and COX-2 were measured by enzyme immunoassay; (**B**) The expression levels of *TNF-α*, *MCP-1*, and *iNOS* mRNA in the colonic epithelium were examined by quantitative real-time RT-PCR using specific primers. The values are expressed as mean ± SD. * $p < 0.01$, ** $p < 0.05$.



2.6. Discussion

Increasing evidence suggests that Mets is involved in the development of CRC, and this continues to be a growing health problem worldwide, especially in developed countries [1–5]. Recent epidemiological studies have suggested that patients with hypertension, a component of Mets [1,2], comprise a high-risk

group for CRC [3–5]. However, appropriate animal models to evaluate hypertension-related colorectal carcinogenesis have not yet been generated.

To our knowledge, the present study provides the first evidence that after administration of AOM, SHRSP and SHRSP-ZF rats, both of which present with hypertension, more readily develop colonic preneoplastic lesions than normotensive WKY rats. In particular, we found that SHRSP rats experience accelerated development of ACF. This is significant because these rats did not exhibit insulin resistance, hyperleptinemia, or dyslipidemia and did not have increased adipose tissue, which are involved in the pathophysiology thought to link Mets to CRC [5–8]. These findings, therefore, suggest that hypertension *per se* might play a critical role in the early events of colorectal carcinogenesis. We have found that the angiotensin converting enzyme inhibitor captopril, an anti-hypertensive drug, significantly prevents the development of ACF in SHRSP-ZF rats [19]. These findings also support our hypothesis that blood pressure elevation *per se* might be directly involved in the early stage of colorectal carcinogenesis. However, in order to test this hypothesis, further studies are needed to establish whether other anti-hypertensive agents, such as AT-II type 1 receptor blockers and calcium channel blockers, can suppress the development of ACF by lowering blood pressure.

Among the pathophysiological disorders associated with hypertension, an increased level of oxidative stress is thought to be particularly important in CRC development [5,6]. Oxidative stress, defined as the overproduction of oxygen species combined with inadequate anti-oxidative defense mechanisms, can result in DNA damage and, consequently, mutations associated with colorectal carcinogenesis [5,20]. In the present study, the hypertensive SHRSP and SHRSP-ZF rats had significantly elevated urine 8-OHdG levels and serum d-ROM levels, which are associated with increased oxidative stress [21]. However, they also had reduced *GPx* and *CAT* mRNA levels, both of which encode antioxidant enzymes, in the colonic epithelium. These findings indicate that both SHRSP and SHRSP-ZF rats are subjected to strong oxidative stress, which might contribute to the development of ACF.

In addition to oxidative stress, the induction of chronic inflammation is also considered to play a critical role in obesity-, diabetes-, and hypertension-related colorectal carcinogenesis [5,6]. In the present study, serum levels of TNF-α and IL-6, as well as colonic expression of *MCP-1* and *iNOS* mRNA, were markedly elevated in SHRSP-ZF obese and diabetic rats. These changes might have been associated with the increase in adipose tissue in SHRSP-ZF rats because excess adipose tissue plays an important role in the exacerbation of systemic inflammation [22,23]. Furthermore, colonic epithelial expression of *TNF*-α mRNA and serum levels of COX-2 were significantly higher in both the hypertensive SHRSP and SHRSP-ZF rats, although the former did not become obese or develop diabetes. These findings are also significant because the dysregulation of TNF-α, a central mediator of chronic inflammatory diseases, and COX-2 have key roles in the stimulation of tumor growth and the progression of carcinogenesis in several tissues, including the colon and rectum [24,25].

Why did the SHRSP rats, which did not exhibit obesity and insulin resistance, experience an acceleration of oxidative stress, exacerbation of chronic inflammation, and development of ACF to the same extent as SHRSP-ZF rats that are both obese and diabetic? One possible explanation is that the dose of AOM (20 mg/kg body weight) used in the present protocol was considerably greater than that needed to induce ACF development in these hypertensive rats. A lower dose of AOM may therefore result in differences in both the number and size of ACF between SHRSP and SHRSP-ZF rats. It is also possible that an increase in the serum level of AT-II, which is the main effector peptide of the

renin-angiotensin system [12,13], might contribute to these phenomena because renin-angiotensin system activation has been implicated in the increase in oxidative stress and the induction of inflammation [11,14,26,27]. Renin-angiotensin system activation induces adipocyte inflammation, as demonstrated by the increased expression of TNF-α and IL-6 in adipose tissue, which in turn is implicated in hypertension [28,29]. In prostate cancer, treatment with AT-II stimulates the secretion of IL-6 and MCP-1 from prostate stromal cells and is associated with the increased proliferation of prostate cancer cells [30]. AT-II also induces the expression of iNOS, an inflammatory marker, along with 8-OHdG in prostate cancer cells [31], suggesting a crosslink between renin-angiotensin system-related inflammation and oxidative stress in cancer tissue.

To date, there is no definitive evidence demonstrating the effectiveness of renin-angiotensin system inhibitors in preventing human malignancies, including CRC, in hypertensive patients [32–35]. However, our findings suggest that targeting hypertension-related metabolic abnormalities, including oxidative stress and chronic inflammation caused by renin-angiotensin system activation, may be an effective strategy to prevent CRC development in patients with Mets, especially those with hypertension. In malignant tissue such as CRC, dysregulation of the renin-angiotensin system is implicated in cancer cell migration, invasion, and metastasis [10,11,13,14,36]. A recent study also showed that treatment with renin-angiotensin system inhibitors could inhibit chemically induced colorectal carcinogenesis in obese and diabetic mice by attenuating chronic inflammation and oxidative stress [37]. In order to test the potential efficacy of renin-angiotensin system inhibitors in preventing CRC development in patients with Mets, additional long-term experiments to evaluate whether these agents can prevent colorectal carcinogenesis in hypertensive rats should be conducted.

3. Experimental Section

3.1. Animals and Chemicals

Five-week-old male SHRSP, SHRSP-ZF, and WKY rats were obtained from Japan SLC (Shizuoka, Japan) and humanely maintained at Gifu University Life Science Research Center in accordance with the Institutional Animal Care Guidelines. The WKY rats are normotensive and not prone to obesity, and thus served as the control group in this study. AOM, which is widely used to mimic sporadic colon carcinogenesis by causing DNA mutations and activating several oncogenic pathways, including the K-*ras* pathway [38,39], was purchased from Wako (Osaka, Japan).

3.2. Experimental Procedure

After 1 week of acclimatization, the 6-week-old rats were divided into 3 groups of 8 rats each. All rats received an intraperitoneal injection of AOM (20 mg/kg body weight) once a week for 2 weeks. The experimental protocol and dose of AOM were based on previous studies using F344, Sprague-Dawley, or Wister rat strains [40,41]. We did not include non-AOM treated WKY rats as negative controls because no ACF was found to develop in these animals in a preliminary experiment. At the end of the experiment (2 weeks after the last injection of AOM), when the rats were 10 weeks of age, systolic and diastolic blood pressures were measured noninvasively using a tail cuff (SOFTRON BP98A; Softron, Tokyo, Japan). All rats were euthanized by CO_2 asphyxiation for colon resection. The third portion of

the excised colons (cecum side) was used to extract RNA, and the remaining part was used to determine the number of ACF [42].

3.3. Enumeration of ACF

The frequency of AOM-induced colonic premalignant lesions, ACF, was determined as previously described [42]. Briefly, the colon samples were fixed with 10% buffered formalin, stained with methylene blue (0.5% in distilled water) for 20 s, and then placed on microscope slides to count the number of ACF. The number of ACF was recorded along with the number of ACs in each focus. The data are expressed per unit area (cm^2).

3.4. RNA Extraction and Quantitative Real-Time Reverse Transcription-Polymerase Chain Reaction Analysis

The epithelial crypts were isolated from colonic tissue [41]. Total RNA was then extracted from the isolated epithelial crypts using the RNAqueous-4PCR kit (Ambion Applied Biosystems, Austin, TX, USA). cDNA was amplified from 0.2 μg of total RNA using the SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). Quantitative real-time reverse transcription-PCR (RT-PCR) analysis was performed using specific primers that amplify *TNF*-α, *MCP-1*, *iNOS*, *GPx*, *CAT*, and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) genes. The sequences of these primers, which were obtained from Primer-BLAST [43], are listed in Table S1. Each sample was analyzed on a LightCycler Nano (Roche Diagnostics, Basel, Switzerland) using FastStart Essential DNA Green Master (Roche Diagnostics). Parallel amplification of *GAPDH* was used as the internal control.

3.5. Clinical Biochemistry

Blood samples from the inferior vena cava were used for chemical analyses. These samples were obtained at the time of euthanasia, prior to which the rats had fasted for 6 h. The serum levels of TNF-α (R&D Systems, Minneapolis, MN, USA), IL-6 (R & D Systems), insulin (Shibayagi, Gunma, Japan), glucose (BioVision Research Products, Mountain View, CA, USA), leptin (Shibayagi), triglyceride (Wako), NEFA (Wako), AT-II (Phoenix Pharmaceuticals, INC, Burlingame, CA, USA), and COX-2 (MyBioSource, San Diego, CA, USA) were determined using an enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer instructions.

3.6. Oxidative Stress Analysis

Urine 8-OHdG levels were determined using an ELISA kit (NIKKEN SEIL, Shizuoka, Japan). Serum levels of hydroperoxide, a marker for oxidative stress, were evaluated using the d-ROM test (FREE Carpe Diem; Diacron s.r.l., Grosseto, Italy) [21].

3.7. Statistical Analysis

All data are presented as mean ± SD and were analyzed using the GraphPad InStat software program version 3.05 (GraphPad Software, San Diego, CA, USA) for Macintosh. One-way analysis of variance

(ANOVA) was used to compare groups. If the ANOVA analysis indicated significant differences, the Tukey-Kramer multiple comparisons test was performed to compare the mean values among the groups. The differences were considered significant when the two-sided p value was less than 0.05.

4. Conclusions

The results of this study indicate that the development of AOM-induced colonic preneoplastic lesions was significantly accelerated in hypertensive rats compared to normotensive rats. This was associated with hypertension-related renin-angiotensin system activation and subsequent induction of oxidative stress and inflammation, suggesting that hypertension plays a critical role in the early stages of CRC.

Conflict of Interest

The authors declare no conflict of interest.

References

1. Alberti, K.G.; Zimmet, P.; Shaw, J.; IDF Epidemiology Task Force Consensus Group. The metabolic syndrome—A new worldwide definition. *Lancet* **2005**, *366*, 1059–1062.
2. Grundy, S.M.; Cleeman, J.I.; Daniels, S.R.; Donato, K.A.; Eckel, R.H.; Franklin, B.A.; Gordon, D.J.; Krauss, R.M.; Savage, P.J.; Smith, S.C., Jr.; *et al.* Diagnosis and management of the metabolic syndrome: An American Heart Association/National Heart, Lung, and Blood Institute Scientific Statement. *Circulation* **2005**, *112*, 2735–2752.
3. Ahmed, R.L.; Schmitz, K.H.; Anderson, K.E.; Rosamond, W.D.; Folsom, A.R. The metabolic syndrome and risk of incident colorectal cancer. *Cancer* **2006**, *107*, 28–36.
4. Stocks, T.; van Hemelrijck, M.; Manjer, J.; Bjorge, T.; Ulmer, H.; Hallmans, G.; Lindkvist, B.; Selmer, R.; Nagel, G.; Tretli, S.; *et al.* Blood pressure and risk of cancer incidence and mortality in the Metabolic Syndrome and Cancer Project. *Hypertension* **2012**, *59*, 802–810.
5. Ishino, K.; Mutoh, M.; Totsuka, Y.; Nakagama, H. Metabolic syndrome: A novel high-risk state for colorectal cancer. *Cancer Lett.* **2012**, doi:10.1016/j.canlet.2012.10.01.
6. Shimizu, M.; Kubota, M.; Tanaka, T.; Moriwaki, H. Nutraceutical approach for preventing obesity-related colorectal and liver carcinogenesis. *Int. J. Mol. Sci.* **2012**, *13*, 579–595.
7. Yuhara, H.; Steinmaus, C.; Cohen, S.E.; Corley, D.A.; Tei, Y.; Buffler, P.A. Is diabetes mellitus an independent risk factor for colon cancer and rectal cancer? *Am. J. Gastroenterol.* **2011**, *106*, 1911–1921.
8. Donohoe, C.L.; Pidgeon, G.P.; Lysaght, J.; Reynolds, J.V. Obesity and gastrointestinal cancer. *Br. J. Surg.* **2010**, *97*, 628–642.
9. De Kloet, A.D.; Krause, E.G.; Woods, S.C. The renin angiotensin system and the metabolic syndrome. *Physiol. Behav.* **2010**, *100*, 525–534.
10. Ager, E.I.; Neo, J.; Christophi, C. The renin-angiotensin system and malignancy. *Carcinogenesis* **2008**, *29*, 1675–1684.
11. Deshayes, F.; Nahmias, C. Angiotensin receptors: A new role in cancer? *Trends Endocrinol. Metab.* **2005**, *16*, 293–299.