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).

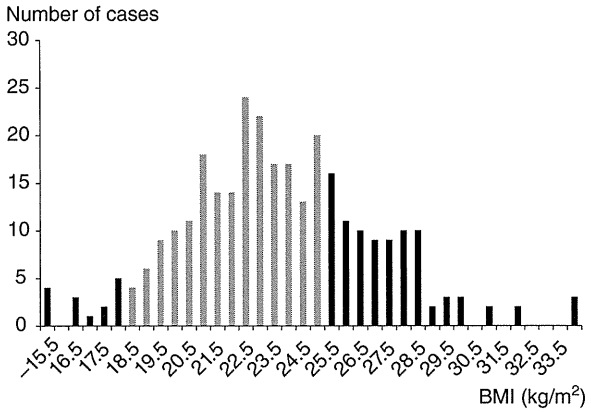

Figure 1 Distribution of body mass index (BMI) in patients with liver cirrhosis. Total number of patients = 294. Obese subjects (BMI ≥ 25) were present in 30.6%, lean ones (18.5 ≤ BMI < 25) were in 64.3% and emaciation (BMI < 18.5) was observed in 5.1%.

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

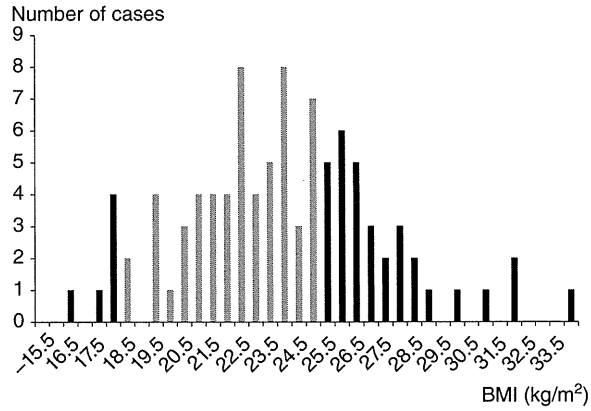

Figure 2 Distribution of body mass index (BMI) in cirrhotic patients without ascites, edema or hepatocellular carcinoma. Total number of patients = 95. Obese subjects (BMI ≥ 25) were present in 33.7%, lean ones (18.5 ≤ BMI < 25) were in 57.1% and emaciation (BMI < 18.5) was observed in 9.2%.

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.

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ORIGINAL PAPER

Hepatocellular carcinoma patients with increased oxidative stress levels are prone to recurrence after curative treatment: a prospective case series study using the d-ROM test

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Abstract
Purpose Oxidative stress plays an important role in liver carcinogenesis. To determine the impact of oxidative stress on the recurrence of stage I/II hepatocellular carcinoma (HCC) after curative treatment, we conducted a prospective case series analysis.
Methods This study included 45 consecutive patients with stage I/II HCC, who underwent curative treatment by surgical resection or radiofrequency ablation at Gifu Municipal Hospital from 2006 to 2007. In these 45 cases, recurrence-free survival was estimated using the Kaplan–Meier method. The factors contributing to HCC recurrence, including the serum levels of derivatives of reactive oxygen metabolites (d-ROM) as an index of oxidative stress, were subjected to univariate and multivariate analyses using the Cox proportional hazards model.
Results The serum levels of d-ROM ($P = 0.0231$), α-fetoprotein (AFP, $P = 0.0274$), and fasting plasma glucose ($P = 0.0400$) were significantly associated with HCC recurrence in the univariate analysis. Multivariate analysis showed that the serum levels of d-ROM (hazard ratio [HR] 1.0038, 95 % confidence interval [CI] 1.0002–1.0071, $P = 0.0392$) and AFP (HR 1.0002, 95 % CI 1.0000–1.0003, $P = 0.0316$) were independent predictors of HCC recurrence. Kaplan–Meier analysis showed that recurrence-free survival was low in patients with high serum d-ROM (≥570 Carr U, $P = 0.0036$) and serum AFP (≥40 ng/dL, $P = 0.0185$) levels.
Conclusions The serum levels of d-ROM and AFP can be used for screening patients with a high risk for HCC recurrence. Patients who show increased levels of these factors require careful surveillance.

Keywords Hepatocellular carcinoma · Oxidative stress · d-ROM · Carcinogenesis

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Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide, accounting for 750,000 annual cases; approximately the same number of people (700,000) die from this malignancy each year (Jemal et al. 2011). HCC development is frequently associated with chronic inflammation and subsequent cirrhosis of the liver induced by persistent infection with hepatitis B virus (HBV) or hepatitis C virus (HCV) (El-Serag 2002). Alcohol consumption, obesity, and related metabolic disorders such as diabetes mellitus are also involved in liver carcinogenesis (El-Serag 2002). The prognosis of patients with HCC is poor because the incidence of recurrence in patients with underlying cirrhosis is very high (Toyama et al. 2008). Therefore, careful surveillance of high-risk groups for HCC and early detection before progression to an advanced stage are important to improve the prognosis of this malignancy. It is therefore a task of pressing urgency to identify useful risk factors for HCC development or recurrence. Male gender, the presence of cirrhosis,

high α-fetoprotein (AFP), large tumor foci, multiplicity of tumors, pathologically high-grade atypia of tumor cells, and the presence of portal venous invasion of tumors are thought to increase the risk for HCC recurrence (Ikeda et al. 1993; Koike et al. 2000). The increased Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) value, which reflects insulin resistance, and high levels of serum leptin, one of the adipokines associated with obesity, are also independent risk factors for HCC recurrence (Imai et al. 2010; Watanabe et al. 2011).

Increased evidence indicates that continuous oxidative stress, which results from the imbalance between the production of reactive oxygen species (ROS) and the antioxidant defense mechanisms, plays a critical role in the development of various human malignancies, including HCC (Sasaki 2006; Valko et al. 2007; Sakurai et al. 2008). As a major site of metabolism, the liver displays high levels of ROS resulting in increased oxidative stress. Oxidative stress is known to induce DNA damage, and accumulation of such genetic damage can eventually contribute to liver carcinogenesis (Sasaki 2006; Valko et al. 2007; Sakurai et al. 2008). HCV infection is associated with elevated levels of ROS and decreased antioxidant levels in patients (Seronello et al. 2007). Oxidative stress has been associated with the development of steatosis and liver tumors in HCV core transgenic mice (Moriya et al. 2001). In addition, increased levels of ROS is also involved in migration, invasion, and metastasis of HCC cells (Hu et al. 2011; Chung et al. 2012). These findings suggest that oxidative stress biomarkers might potentially be useful for predicting the development and recurrence of HCC in patients with chronic liver disease. Clinical studies using liver specimens obtained by biopsy or surgery have shown the predictive power of oxidative stress biomarkers on HCC development (Chuma et al. 2008; Tanaka et al. 2011). However, serum oxidative stress biomarkers predictive for recurrence after curative treatment for HCC have not been investigated.

Quantification of derivatives of reactive oxygen metabolites (d-ROM) is a simple method for detecting hydroperoxide levels (Trotti et al. 2002), and clinical trials have shown that the d-ROM test is useful for evaluating oxidative stress (Trotti et al. 2002; Hirose et al. 2009; Sugiura et al. 2011). In this study, we measured the serum d-ROM level in patients with HCC and designed a prospective case series analysis to examine the recurrence-free survival in consecutive patients with stage I/II HCC who received curative treatment by surgical resection or radiofrequency ablation (RFA), stratified according to the serum d-ROM level. Thus, the aim of the present study was to determine whether the d-ROM test is useful as a marker of oxidative stress for evaluating HCC recurrence risk in the clinical setting.

Patients and methods

Patients

We evaluated 45 consecutive primary HCC patients in Gifu Municipal Hospital from 2006 to 2007, all of whom met the following criteria: tumor stage classified as I or II and surgical resection or RFA as the initial treatment. Tumor stage was defined according to the staging system of the Liver Cancer Study Group of Japan (2010). HCC nodules were detected using imaging modalities including dynamic computed tomography (CT), dynamic magnetic resonance imaging (MRI), and abdominal arteriography. HCC was diagnosed from a typical hypervascular tumor stain on angiography and typical dynamic study findings of enhanced staining in the early phase and attenuation in the delayed phase.

Treatment, follow-up, and determination of recurrence

One patient was treated with surgical resection, 41 with RFA, and 3 with RFA after transarterial chemoembolization. The selection criteria for the initial treatments were determined according to the Clinical Practice Guidelines for HCC by the Japan Society of Hepatology (Clinical Practice Guidelines for Hepatocellular Carcinoma—The Japan Society of Hepatology 2009 update 2010). The response to treatment was defined as complete response when dynamic CT or MRI showed complete disappearance of the HCC imaging characteristics in all target lesions, according to the Response Evaluation Criteria in Cancer of the Liver (Kudo et al. 2010).

Patients were thereafter followed up on an outpatient basis by assessing the levels of serum tumor markers such as AFP and proteins induced by vitamin K absence or antagonist-II (PIVKA-II) every month and by using imaging modalities such as abdominal ultrasonography, dynamic CT scanning, or dynamic MRI every 3 months. Recurrent HCC was defined as the appearance of distant lesions to exclude local recurrence. Consequently, recurrent HCC was further classified into multicentric occurrence or intrahepatic metastasis by CT images according to the definition by the Liver Cancer Study Group of Japan (Liver Cancer Study Group of Japan 2010). The follow-up period was defined as the interval from the date of initial treatment until the date of diagnosis of recurrence or until March 2012 if HCC did not recur.

Oxidative stress assay

Before curative treatment, oxidative stress was assessed by measuring the serum hydroperoxide concentration according to the d-ROM test (Diacron srl, Grosseto, Italy) by

Table 1 Baseline demographic and clinical characteristics

Variables	$n = 45$
Sex (male/female)	30/15
Age (years)	72 [50–82]
BMI (kg/m^2)	22.8 [15.6–33.5]
Etiology (B/C/B + C/other)	3/40/1/1
Follow-up period (days)	1,707 [305–2,231]
d-ROM (Carr U)	496 [295–869]
Child-Pugh classification (A/B/C)	33/12/0
ALB (g/dL)	3.5 [2.6–4.5]
ALT (IU/L)	51 [12–100]
T-Bil (mg/dL)	1.0 [0.5–3.7]
PLT ($\times 10^4/\mu$L)	9.8 [3.6–19.5]
PT (%)	71 [50–100]
FPG (mg/dL)	100 [41–224]
HbA_{1c} (%)[a]	5.7 [4.0–9.8]
AFP (ng/dL)	32.5 [1.7–16,931]
PIVKA-II (mAU/mL)	23.0 [5–1,860]
Stage (I/II)	21/24
Tumor size (cm)	1.7 [1.0–5.3]
Tumor number (1/2/3/4)	36/6/1/2
Portal vein invasion (yes/no)	0/45
Initial treatment for HCC (resection/RFA/TACE + RFA)	1/41/3

Values are presented as median [range]. *BMI* body mass index, *d-ROM* derivatives of reactive oxygen metabolites, *ALB* albumin, *ALT* alanine aminotransferase, *T-Bil* total bilirubin, *PLT* platelet count, *PT* prothrombin time, *FPG* fasting plasma glucose, *HbA_{1c}* hemoglobin A1c, *AFP* α-fetoprotein, *PIVKA-II* protein induced by vitamin K absence or antagonists-II, *RFA* radiofrequency ablation, *TACE* transarterial chemoembolization

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

using a free radical elective evaluator, FREE (Diacron srl), as described previously (Trotti et al. 2002; Hirose et al. 2009; Sugiura et al. 2011).

Statistical analysis

Recurrence-free survival was estimated using the Kaplan–Meier method, and differences between curves were evaluated using the logrank test. Baseline characteristics were compared using the Student's t test for continuous variables or the χ^2 test for categorical variables. Eleven possible predictors for HCC recurrence after initial curative treatment were selected as follows: sex, age, body mass index (BMI), Child-Pugh classification, serum albumin level, platelet count, fasting plasma glucose (FPG), serum AFP level, serum PIVKA-II level, tumor stage, and the serum d-ROM level. Parameters determined to be significant according to univariate analysis were then subjected to multivariate analysis using the Cox proportional hazards model. Receiver operating characteristic (ROC) analysis was used to identify the cut-off values for d-ROM and AFP that would best predict HCC recurrence. Statistical significance was defined as $P < 0.05$.

Results

Baseline characteristics and laboratory data of patients

The baseline characteristics and laboratory data of the 45 patients (30 men and 15 women, median age: 72 years) are shown in Table 1. The median follow-up period was 1,707 days (range 305–2,231 days). Thirty-three patients were classified as Child-Pugh class A, 12 patients as class B, and none as class C. The median d-ROM level of all the patients with HCC was 496 Carr U (range 295–869 Carr U).

Possible risk factors for HCC recurrence

In all 45 curative cases of stage I/II HCC, 41 patients experienced recurrence in the liver and 2 patients exhibited distant metastasis; 1 in the lung and the other in the bone. The 1-year, 3-year, and 5-year recurrence-free survival rates in the 45 patients were 60, 29, and 7 %, respectively (Fig. 1a). Among 41 cases that caused intrahepatic recurrence of HCC, 36 cases were diagnosed as multicentric occurrence and the others (5 cases) were as intrahepatic metastasis, respectively.

At first, we analyzed possible risk factors for total recurrence including both multicentric occurrence and intrahepatic metastasis by the Cox proportional hazards model using the 11 variables listed in Table 2. The serum d-ROM level (hazard ratio [HR] 1.0036, 95 % confidence interval [CI] 1.0005–1.0070, $P = 0.0231$), serum AFP level (HR 1.0001, 95 % CI 1.0000–1.0002, $P = 0.0274$), and FPG (HR 1.0008, 95 % CI 1.0004–1.0157, $P = 0.0400$) were significantly associated with HCC recurrence in univariate analysis. Among these variables, multivariate analysis indicated that serum levels of d-ROM (HR 1.0038, 95 % CI 1.0002–1.0071, $P = 0.0392$) and AFP (HR 1.0002, 95 % CI 1.0000–1.0003, $P = 0.0316$) were independent predictors of HCC recurrence (Table 3).

The cut-off values of d-ROM (570 Carr U) and AFP (40 ng/dL) for the prediction of HCC recurrence were determined by ROC analysis. Kaplan–Meier analysis showed that recurrence-free survival was lower in patients with high serum d-ROM levels (≥570 Carr U, $P = 0.0036$) (Fig. 1b) and in those with high serum AFP levels (≥40 ng/dL, $P = 0.0185$) (Fig. 1c). Table 4 shows the baseline characteristics and laboratory data of patients

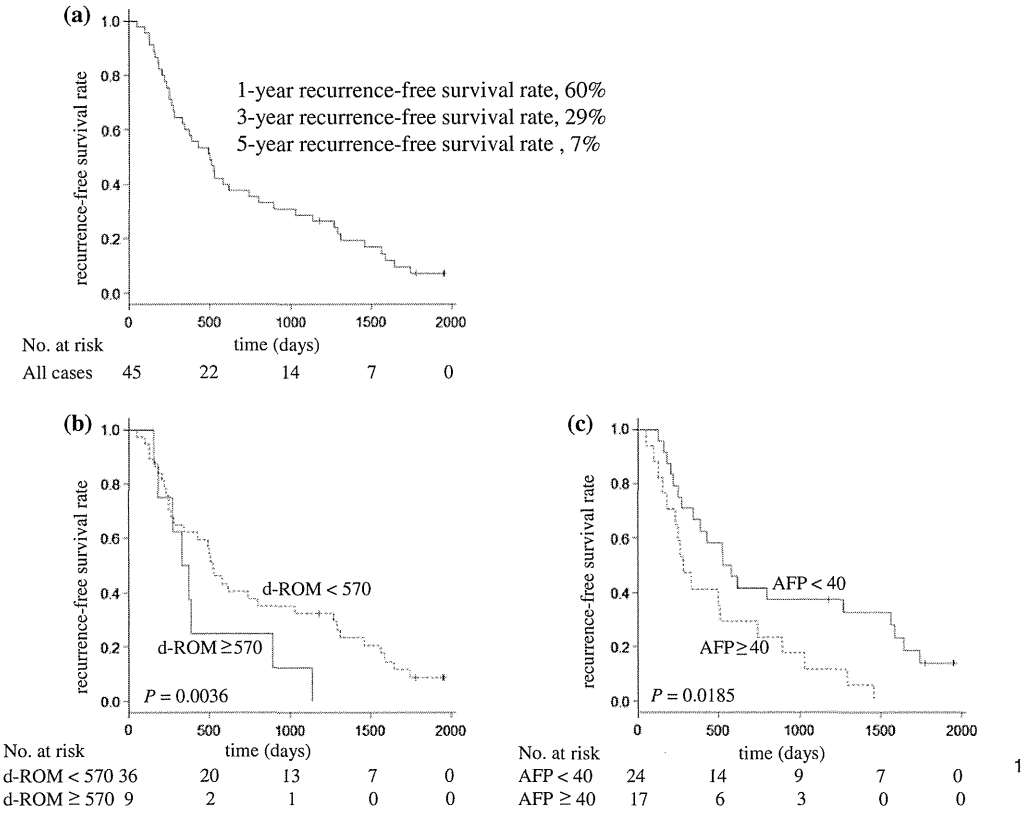

Fig. 1 Kaplan–Meier curves for recurrence-free survival in (**a**) all patients and in subgroups of patients, divided according to (**b**) d-ROM levels or (**c**) serum AFP levels

Table 2 Univariate analyses of possible risk factors for hepatocellular carcinoma recurrence according to a Cox proportional hazards model

Variable	HR	95 % CI		*P* value
		Lower limit	Upper limit	
Sex (male vs. female)	0.9142	0.4810	1.8307	0.7917
Age (years)	1.0086	0.9714	1.0473	0.6522
BMI (kg/m^2)	0.9878	0.9082	1.0696	0.7695
Child-Pugh classification (B vs. A)	0.9707	0.4643	1.8843	0.9329
ALB (g/dL)	0.9496	0.4600	1.9744	0.8893
PLT ($\times 10^4$/mL)	0.9817	0.9031	1.0613	0.6507
FPG (mg/dL)	1.0008	1.0004	1.0157	0.0400
AFP (ng/dL)	1.0001	1.0000	1.0002	0.0274
PIVKA-II (mAU/mL)	1.0005	0.9996	1.0012	0.2149
Stage (II vs. I)	1.1968	0.6468	2.2348	0.5664
d-ROM (Carr U)	1.0036	1.0005	1.0070	0.0231

CI confidence interval, *HR* hazard radio, *BMI* body mass index, *ALB* albumin, *PLT* platelets, *FPG* fasting plasma glucose, *AFP* α-fetoprotein, *PIVKA-II* protein induced by vitamin K absence or antagonists-II, *d-ROM* derivatives of reactive oxygen metabolites

divided on the basis of the serum d-ROM concentration (<570 Carr U and ≥ 570 Carr U). No significant differences were noted between the 2 subgroups.

The serum d-ROM levels were not significantly correlated with any clinical factors associated with hepatic functional reserve (serum total bilirubin, serum albumin,

Table 3 Multivariate analyses of possible risk factors for hepatocellular carcinoma recurrence according to a Cox proportional hazards model

Variable	HR	95 % CI		*P* value
		Lower limit	Upper limit	
FPG (mg/dL)	1.0004	0.9961	1.0126	0.2699
AFP (ng/dL)	1.0002	1.0000	1.0003	0.0316
d-ROM (Carr U)	1.0038	1.0002	1.0071	0.0392

CI confidence interval, *HR* hazard radio, *FPG* fasting plasma glucose, *AFP* α-fetoprotein, *d-ROM* derivatives of reactive oxygen metabolites

serum alanine aminotransferase, prothrombin time, and platelet count) and HCC (tumor size, AFP, and PIVKA-II) (Table 5). In addition, the d-ROM levels did not show significant relationships between the clinical factors for diabetes, including FPG, HbA_{1c}, fasting immunoreactive insulin, and HOMA-IR (Table 5), although several studies have reported that oxidative stress increases with the presence of diabetes and that the d-ROM level is correlated with diabetic factors (Vassalle et al. 2008; Hirose et al. 2009; Sugiura et al. 2011). Further, no significant differences in the median values of d-ROM were noted between (1) the Child-Pugh class A group (496 Carr U, range 295–869 Carr U) and the Child-Pugh class B group (500 Carr U, range 314–589 Carr U) and (2) the stage I group (516 Carr U, range 295–858 Carr U) and the stage II group (500 Carr U, range 314–869 Carr U), suggesting that d-ROM values were not associated with hepatic functional reserve, tumor factors, or the presence of diabetes in this study. These findings indicate that an increased d-ROM value was independently related to the recurrence of HCC.

A separate analysis of 36 multicentric occurrence cases showed an inverse correlation between the serum d-ROM levels and the recurrence-free period, but the significance was marginal ($P = 0.0512$) due to the small number of patients (data not shown). In addition, intrahepatic metastasis cases ($N = 5$) showed a higher d-ROM levels (median 614 Carr U, range 496–869 Carr U) than non-recurrent cases ($N = 4$, median 474 Carr U, range: 461–509 Carr U) ($P = 0.112$).

Discussion

Increasing evidence suggests that oxidative stress plays a critical role in liver carcinogenesis (Sasaki 2006; Sakurai et al. 2008). Elevation of ROS can cause oxidative damage to important cellular macromolecules such as DNA, proteins, and lipids (Valko et al. 2007). Excessive ROS also disrupts the cell signaling pathways that are involved in cell growth and survival, leading further to the advanced stage of carcinogenesis, and cancer promotion and progression (Dreher and Junod 1996; Carmeliet 2000).

Table 4 Baseline demographic and clinical characteristics of patients classified according to the d-ROM level

	d-ROMs < 570 ($n = 36$)	d-ROMs ≥ 570 ($n = 9$)	*P* value
Sex (male/female)	24/12	6/3	1.0000
Age (years)	72 [50–82]	69 [58–76]	0.5325
BMI	22.5 [15.6–33.5]	23.0 [20.1–27.0]	0.7965
Etiology (B/C/B + C/other)	3/31/1/1	0/9/0/0	0.4968
Follow-up period (days)	1,712 [458–2,231]	1,643 [305–2,146]	0.2350
Child-Pugh classification (A/B)	26/10	7/2	0.7323
ALB (g/dL)	3.5 [2.6–4.5]	3.5 [3.0–4.4]	0.9312
ALT (IU/L)	48 [12–100]	53 [22–73]	0.5621
T-Bil (mg/dL)	1.0 [0.5–3.7]	1.0 [0.6–1.5]	0.8532
PLT ($\times 10^4/\mu L$)	10.4 [3.8–19.5]	6.6 [3.6–18.8]	0.0843
PT (%)	70 [50–100]	77 [59–90]	0.2293
FPG (mg/dL)	99 [41–224]	104 [83–140]	0.6272
HbA_{1c} (%)[a]	5.7 [4.0–9.8]	5.3 [4.8–7.0]	0.6447
AFP (ng/dL)	31 [1.7–16,931]	33 [16.7–210]	0.5130
PIVKA-II (mAU/mL)	19.5 [5–1,540]	57.0 [7–1,860]	0.2822
Stage (I/II)	17/19	4/5	0.8811
Initial treatment for HCC (resection/RFA/TACE + RFA)	1/32/3	0/9/0	0.3905

Values are presented as median [range]. *BMI* body mass index, *ALB* albumin, *ALT* alanine aminotransferase, *T-Bil* total bilirubin, *PLT* platelets, *PT* prothrombin time, *FPG* fasting plasma glucose, *HbA_{1c}* hemoglobin A1c, *AFP* α-fetoprotein, *PIVKA-II* protein induced by vitamin K absence or antagonists-II, *HCC* hepatocellular carcinoma

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

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.

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Conflict of interest The authors declare no conflict of interest.

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Article

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

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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.

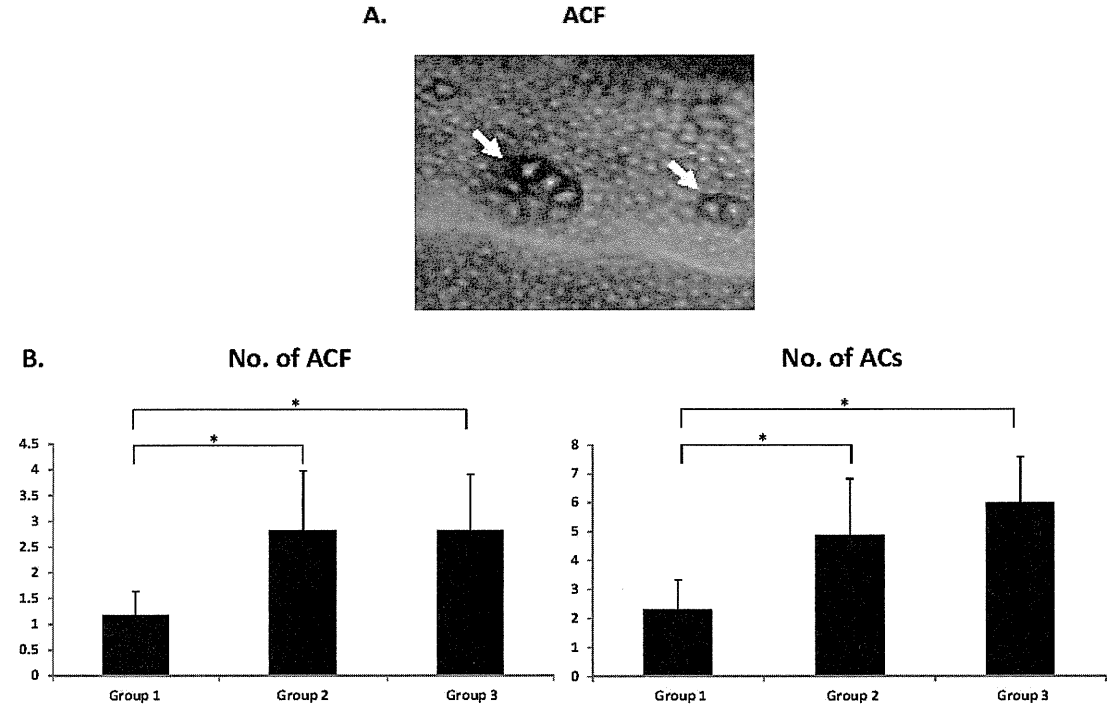
	Glucose (mg/dL)	Insulin (μIU/mL)	Quicki	Leptin (pg/mL)	NEFA (mEq/L)	Triglyceride (mg/dL)	Angiotensin II (ng/mL)
Group 1	85.4 ± 11.7	15.81 ± 0.35	0.313 ± 0.010	11.2 ± 3.6	0.459 ± 0.03	27.1 ± 7.4	352.6 ± 38.1
Group 2	83.5 ± 12.3	17.00 ± 1.39	0.320 ± 0.008	12.2 ± 3.4	0.419 ± 0.05	39.6 ± 14.1	494.4 ± 75.6 [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.

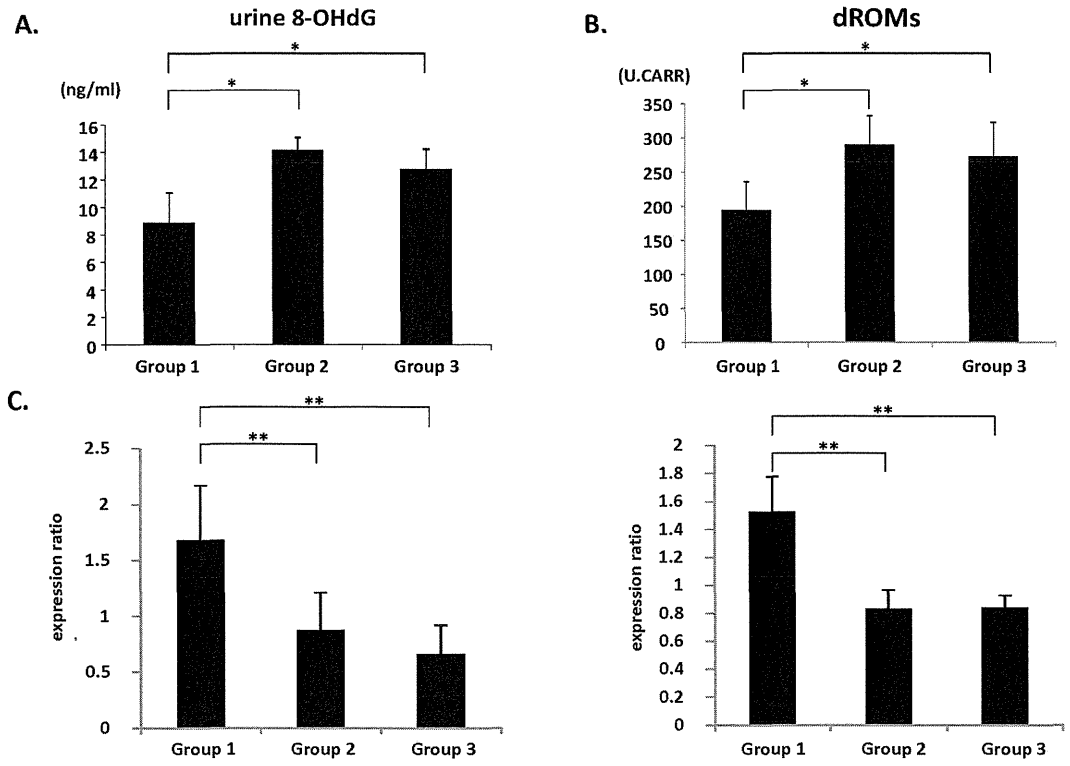
Figure 1. ACF developed in the SHRSP, SHRSP-ZF, and WKY rats that received AOM. (**A**) Representative morphology of ACF (arrows) induced by AOM stained with methylene blue in Group 2. Magnification, 40×; (**B**) Average number of ACF and ACs (/cm^2). Group 1: WKY rats, Group 2: SHRSP rats, and Group 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. * $p < 0.01$, ** $p < 0.01$.

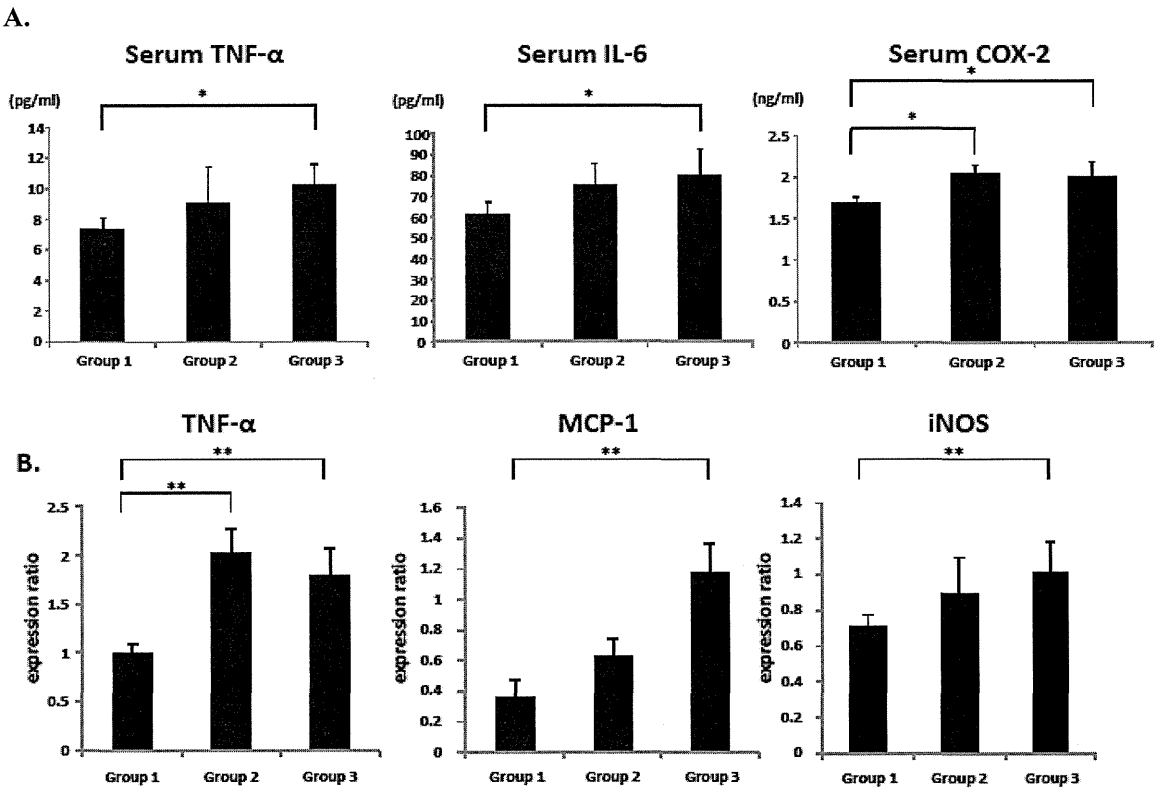


2.5. Serum and Colonic Epithelial Expression of Inflammatory Markers

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