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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Increased hepatic oxidative DNA damage in patients with nonalcoholic steatohepatitis who develop hepatocellular carcinoma

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Abstract
Background The rate of onset of hepatocellular carcinoma (HCC) in patients with nonalcoholic steatohepatitis (NASH) has been reported recently to be comparable to that of patients with chronic hepatitis C. However, the precise mechanism contributing to carcinogenesis in the former remains unclear. Although increased oxidative stress is presumed to play a role in carcinogenesis in patients with NASH, this relationship remains to be directly proven. In this study, we investigated the involvement of oxidative DNA damage in hepatocarcinogenesis in patients with NASH.
Methods Patients with nonalcoholic fatty liver disease who were treated at our university hospital were eligible for enrolment in the study($n = 49$). The study cohort included 30 patients with NASH without HCC (NASH without HCC), six HCC patients with NASH (NASH–HCC), and 13 patients with simple steatosis. Quantitative immunohistochemistry with a KS-400 image analyzing system was used for 8-hydroxy-2′-deoxyguanosine (8-OHdG) detection.
Results The 8-OHdG content in the liver tissue of NASH–HCC patients was significantly different from that in the other patients. The median immunostaining intensity was 8.605 in the NASH–HCC cases, which was significantly higher than that in the cases of NASH without HCC (4.845; $P = 0.003$). Multivariate analysis using hepatic 8-OHdG content as a factor in addition to age and fasting blood sugar revealed a significant difference in clinicopathological factors between NASH–HCC and NASH without HCC cases. Old age ($P = 0.015$) and high relative immunostaining intensity for intrahepatic 8-OHdG ($P = 0.037$) were identified as independent factors.
Conclusions 8-OHdG content in liver tissue may serve a marker of oxidative stress and could be a particularly useful predictor of hepatocarcinogenesis.

Keywords Nonalcoholic steatohepatitis · Nonalcoholic fatty liver disease · Hepatocellular carcinoma · Oxidative DNA damage · 8-Hydroxy-2′-deoxyguanosine

Abbreviations

ALD	Alcoholic liver disease
APRI	Aspartate aminotransferase to platelet ratio index
4-HNE	4-Hydroxy-2-nonenal
NAFLD	Nonalcoholic fatty liver disease
NAS	NAFLD activity score
NASH	Nonalcoholic steatohepatitis
NBNC-HCC	Non-B non-C hepatocellular carcinoma
8-OHdG	8-Hydroxy-2′-deoxyguanosine
ROS	Reactive oxygen species
SS	Simple steatosis

Introduction

Hepatocellular carcinoma (HCC) is the fifth leading cause of cancer and the third leading cause of cancer-related deaths [1, 2]. The major risk factors for HCC are infection with hepatitis B virus (HBV) or hepatitis C virus (HCV), although 30–40 % of patients with HCC do not show chronic infection

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with HBV or HCV [3]. Recently, HCC onset rates in patients with nonalcoholic steatohepatitis (NASH), a severe form of nonalcoholic fatty liver disease (NAFLD), have been reported. Ascha et al. [4] found a yearly cancer onset rate of 2.6 % in patients with NASH-related liver cirrhosis and 4.0 % in patients with HCV-related liver cirrhosis; the rate in NASH cases was comparable to that in chronic hepatitis C cases.

Although old age and liver fibrosis have been reported as risk factors for HCC in patients with NASH [4–9], the precise mechanism contributing to hepatocarcinogenesis in such patients remains unclear. It has been reported that increased oxidative stress arising from a variety of hepatocyte-damaging factors is involved in the onset of NASH [10–12]. Although such mechanisms are presumed to be related to carcinogenesis in NASH cases, their involvement remains to be directly proven.

The DNA oxidative stress marker 8-hydroxy-2′-deoxyguanosine (8-OHdG) induces a G:C to T:A mutation by replacing cytosine with adenine for coupling with guanine via oxidation at the C8 position [13–15]. We have previously demonstrated that a close relationship exists between oxidative DNA damage, hepatocarcinogenesis, and hepatic iron overload in patients with chronic HCV infection [16]. However, the significance of 8-OHdG content in HCC tissue from patients with NASH has not yet been reported.

In the study reported here, we compared the amount of 8-OHdG in cancerous and non-cancerous tissues in HCC patients with NASH (NASH–HCC) with that in patients with HCC due to other causes; namely, those with simple steatosis (SS) and those with NASH without HCC (NASH without HCC). We also investigated the involvement of oxidative DNA damage in hepatocarcinogenesis in patients with NASH.

Methods

Patients and tissue samples

A total of 49 patients with NAFLD underwent liver biopsy or liver resection between February 2003 and January 2012 at our hospital, at which time liver tissue samples were obtained. Of these patients, 36 were diagnosed, based on histological findings, with NASH and six with HCC. In the HCC cases, NASH and HCC were diagnosed at the same time. In addition, resected liver tissue samples from patients treated at our hospital for chronic hepatitis B (11 patients; HBV–HCC), chronic hepatitis C (17 patients; HCV–HCC), alcoholic liver disease (11 patients; ALD–HCC), HCC due to unknown causes (9 patients), and liver hemangioma and focal nodular hyperplasia who underwent hepatectomy (8 patients) were employed as controls for analyzing the amount of 8-OHdG in the liver tissue. In all patients, with the exception of the latter eight patients, non-cancerous liver tissue samples for the comparison of 8-OHdG were obtained by liver biopsy. This study was carried out after approval was obtained from the ethical committee of our hospital, and written informed consent was obtained after detailed explanation in all cases according to the Declaration of Helsinki.

Definitions and criteria

In all cases, present and past medical histories were obtained during interviews, and then physical examinations were performed. The diagnosis of NASH was carried out according to the following criteria: (1) patient was not an alcoholic (ethanol intake of $\leq$20 g/day); (2) pathological findings, including presence of hepatic steatosis and inflammation with hepatocyte injury (ballooning) with or without fibrosis; (3) exclusion of liver diseases due to other causes (viral hepatitis, autoimmune hepatitis, drug-induced hepatitis, primary sclerosing cholangitis, primary biliary cirrhosis, and metabolic disease such as Wilson's disease and hemochromatosis) [17–19].

ALD was diagnosed according to the following criteria: (1) ethanol consumption of $\geq$80 g/day for $\geq$5 years; (2) exclusion of liver disease due to other causes [20]. Non-B non-C (NBNC) HCC was defined as HCC from the hepatitis B surface antigen (HBsAg)-negative and HCV antibody-negative background, while HBV–HCC and HCV–HCC were defined as HCC from the HBsAg-positive and HCV antibody-positive background, respectively. Of the NBNC–HCC cases, HCC in patients with liver disease that was not categorized as NASH or ALD were defined as undetermined HCC (undetermined-HCC). The blood chemistry parameters analyzed included fasting blood sugar (FBS), fasting insulin, glycated hemoglobin (HbA1c), total cholesterol, triglyceride, uric acid, C-reactive protein (CRP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl transpeptidase (γ-GTP), platelet count, type IV collagen, serum iron, serum ferritin, and total iron-binding capacity.

Diabetes was diagnosed according to the classification of the Japan Diabetes Society, namely, FBS at $\geq$126 mg/dL, random blood sugar or 2-h blood sugar determined by oral glucose tolerance test at $\geq$200 mg/dL, HbA1c (NGSP test) at $\geq$6.5 %, or treatment with an oral hyperglycemic agent or insulin [21].

Hypertension was diagnosed according to the classification of the Japanese Society of Hypertension, i.e., systolic blood pressure of $\geq$130 mmHg, diastolic blood pressure of $\geq$85 mmHg, or treatment with an antihypertensive drug [22]. Activity (Grade 1–3) and fibrosis (Stage 1–4) of NASH were diagnosed pathologically according to the classification by Brunt [23].

HCC was diagnosed histologically or when typical image findings were recognized by at least two imaging modalities, according to the practice guidelines of the American Association for the Study of Liver Diseases [24].

Semi-quantitative assessment of tissue iron accumulation (Perl's Prussian blue staining)

Perl's Prussian blue staining is a classic semiquantitative method to assess iron accumulation within tissues. The degree of iron accumulation in liver tissue was assessed using criteria previously published by Rowe et al. [25]. Briefly, Grade 0 was assessed if granules were absent or barely discernible at 40× magnification; Grade 1, if barely discernible at 20×; Grade 2 if granules were seen at 10×; Grade 3 if granules were seen at 2×; Grade 4+ if granules could be seen with the naked eye.

Immunohistochemical determination of hepatic 8-OHdG

Immunohistochemical analysis of formalin-fixed, Paraffin-embedded tissue samples was performed using an avidin–biotin–peroxidase complex technique after microwave antigen retrieval, as described previously [16]. Sections (thickness 4 μm) were successively treated with blocking solution, 1 μg/mL anti-8-OHdG monoclonal antibody (Japan Institute for the Control of Aging, Fukuroi, Japan), or normal mouse immunoglobulin G (IgG; Dako, Glostrup, Denmark), biotinylated secondary antibody, and a peroxidase–avidin complex (Envision Plus kit; Dako Japan Co. Ltd, Kyoto, Japan). The intensity of 8-OHdG immunostaining in the sections was assessed by using an AxioCam photomicroscope and KS-400 image analyzing system (Carl Zeiss Vision GmbH, Hallbergmoos, Germany). A microscopic image of each liver section was imported into the KS-400 system, and brown-stained tissues, which represented positively stained nuclei of hepatocytes corresponding to 8-OHdG immunoreactivity, were converted into a 255-graded gray scale. The average gray scale intensity of each sample was calculated by using the KS-400 image analyzing program and was represented as the ratio relative to each sequential section immunostained by normal mouse IgG. At least three periportal and three perivenous zones were examined in each section, and the average of the scores was determined.

Semi-quantitative assessment of 4-hydroxy-2-nonenal immunostaining

Formalin-fixed paraffin embedded specimens were stained (according to the manufacturer's protocol) using a Bond-max™ fully automated staining system (Leica Microsystems GmbH, Wetzlar, Germany) with 0.1 μg/mL anti-4-hydroxy-2-nonenal (HNE) immunostaining monoclonal antibody (Japan Institute for the Control of Aging). The intensity of HNE staining was scored from 0 to 3, as previously described [26], with minor modifications, where; 0 = none, 1 = mild (punctuated labeling), 2 = moderate (dense labeling in a few cells), and 3 = strong (dense and homogenous labeling in numerous cells). At least three random fields per sample were examined, and the average of the scores was determined as the HNE index.

Statistical analysis

Two groups were compared by using the Fisher exact test for categorical data and the Wilcoxon Mann–Whitney U test for quantitative data. The Spearman rank correlation was used to quantify the association between continuous or ordered categorical variables. Logistic regression analysis was used to identify significant risk factors for hepatocarcinogenesis in NASH patients. P values of <0.05 from two-sided tests were considered to be significant.

Results

Comparison of the 8-OHdG content in cancerous and non-cancerous tissues in different liver disease backgrounds

The results for the comparison of 8-OHdG content in cancerous tissue from patients with different background diseases are shown in Fig. 1. There was no significant difference between HCV–HCC and NASH–HCC cases (relative immunohistochemical staining intensity: 6.137 vs. 11.004; $P = 0.085$), whereas the 8-OHdG content in cancerous tissue was significantly greater in NASH–HCC cases than in HBV–HCC cases (11.004 vs. 1.965; $P = 0.002$). Moreover, 8-OHdG content in cancerous tissue was significantly higher in NASH–HCC cases versus ALD–HCC cases (11.004 vs. 2.802; $P = 0.003$) and undetermined-HCC cases (11.004 vs. 2.642, $P = 0.003$).

The results for the comparison of 8-OHdG content in non-cancerous liver tissue in patients with different background liver diseases are shown in Fig. 2. The 8-OHdG content in non-cancerous liver tissue was comparable to that in cancerous tissue. There was no significant difference in 8-OHdG content between patients with HCV–HCC and NASH–HCC (relative immunohistochemical staining intensity 5.820 vs. 8.605, respectively; $P = 0.055$), whereas the 8-OHdG content in non-cancerous tissue was significantly greater in NASH–HCC patients versus HBV–HCC patients (8.605 vs. 2.605, respectively; $P = 0.002$). Moreover, the level in NASH–HCC patients was significantly greater than that in

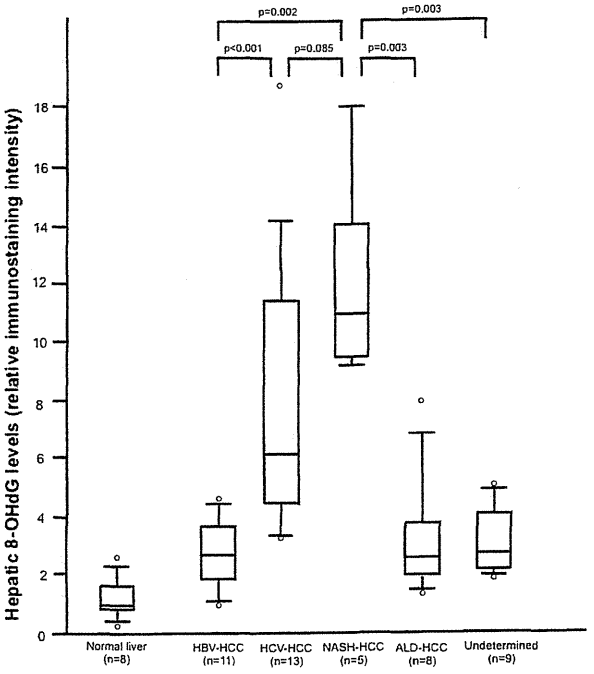

Fig. 1 Comparison between hepatic 8-hydroxy-2′-deoxyguanosine (*8-OHdG*) levels in cancerous tissues in hepatocellular carcinoma (*HCC*) patients with hepatitis B virus (*HBV–HCC*), hepatitis C virus (*HCV–HCC*), alcoholic liver disease (*ALD–HCC*), and nonalcoholic steatohepatitis (*NASH–HCC*; cancerous tissue) and in those with undetermined HCC (*undetermined*). Relative staining intensity of hepatic 8-OHdG was quantified by computer-assisted image analysis. The results are shown as *box plot profiles*, with the *bottom* and *top edges* of the *boxes* representing the 25th and 75th percentiles, respectively. Median values are shown by the *line within the box*. Values outside the top or bottom 95th percentiles are shown individually. *P* values for comparison of normal liver with HBV–HCC, HCV–HCC, NASH–HCC, ALD–HCC, and undetermined HCC cases were 0.013, <0.001, 0.003, 0.009, and 0.002, respectively

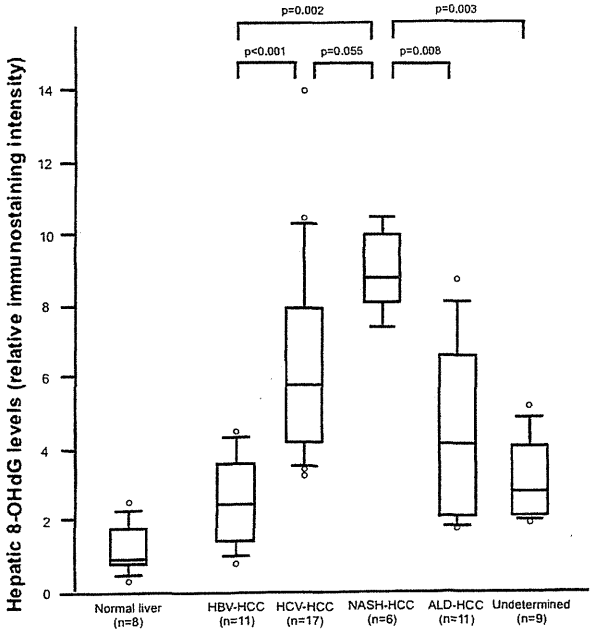

Fig. 2 Comparison between hepatic 8-OHdG levels in patients with HBV–HCC, HCV–HCC, ALD–HCC, undetermined HCC, and NASH–HCC (non-cancerous tissue). Relative staining intensity of hepatic 8-OHdG was quantified by computer-assisted image analysis. The results are shown as *box plot profiles*, with the *bottom* and *top edges* of the *boxes* representing the 25th and 75th percentiles, respectively. Median values are shown by the *line within the box*. Values outside the top or bottom 95th percentiles are shown individually. *P* values for comparison of normal liver with HBV–HCC, HCV–HCC, NASH–HCC, ALD–HCC, and undetermined HCC cases were 0.017, <0.001, 0.003, 0.001, and 0.002, respectively

ALD–HCC patients (8.605 vs. 4.075, respectively; $P = 0.008$) and patients with undetermined HCC (8.605 vs. 2.783, respectively; $P = 0.003$).

Clinical characteristics of the NAFLD patients

Since the intrahepatic 8-OHdG content was significantly increased both in cancerous and non-cancerous tissues in NASH–HCC cases versus HCV–HCC cases, we speculated that the accumulation of 8-OHdG was potentially involved in the transition to carcinogenesis from NASH. We then compared intrahepatic 8-OHdG content between 13 SS and 36 NASH cases (6 with and 30 without HCC onset).

Background factors in these cases are shown in Table 1. Of the six HCC cases, there were three cases of females with HCC and three cases of males with HCC. The median body mass index (BMI) was 25.4 (range 23.5–28.9) kg/m^2, which suggested a tendency for obesity in these patients. All cases had hypertension, and diabetes was concomitantly observed in many as 67 % of the patients. Fibrosis of liver tissue was observed as follows: Brunt's classification Stage 0, one case; Stage 1, one case; Stage 2, one case; Stage 3, three cases. HCC also developed in relatively undeveloped fibrosis cases. Tumor stages at the time of diagnosis (UICC-TNM Classification, Ver. 7) were as follows: Stage I, two cases; Stage II, one case; Stage IIIA, three cases. HCC was often observed in relatively advanced cases. Univariate analysis revealed that there was a significant difference in factors related to insulin resistance and fibrosis between patients with SS and NASH. After gender bias was taken into account and values adjusted by division with the upper limit of normal were compared, serum ferritin levels showed a statistically significant difference. The median patient age in the HCC onset cases was 73 (range 72–80) years, which was significantly older than the median age of 53 (range 24–77) years in cases without HCC onset ($P < 0.001$). In addition, the median FBS was 154 (range 109–212) mg/dL in the HCC onset cases, which was significantly higher than the

median FBS of 107 (range 82–233) mg/dL in cases without HCC onset ($P = 0.012$).

Pathological findings of the NAFLD patients

There was a significant difference in intralobular inflammation ($P < 0.001$), ballooning ($P < 0.001$), and NAFLD activity score (NAS) ($P < 0.001$), as well as in the grade ($P < 0.001$) and stage ($P < 0.001$) according to Brunt's classification, between patients with SS and those with NASH without HCC. There were no significant differences in any of the clinical parameters between patients with NASH–HCC and those with NASH without HCC (Table 2).

8-OHdG and 4-HNE content in liver tissue from NALFD patients

The 8-OHdG content in liver tissue in NASH–HCC patients was significantly different from that patients with normal liver tissue and those with SS and NASH without HCC. Each representative staining pattern is shown in Fig. 3. The median 8-OHdG content was 8.605 (range 7.241–10.343) in NASH–HCC cases, which was significantly greater than the median of 4.845 (range 1.794–9.683) in NASH without HCC cases ($P = 0.003$) (Fig. 4).

Consistent with previous reports [27], the 4-HNE index (a lipid peroxidation marker) was significantly higher in

Table 1 Clinical characteristics of patients with NASH–HCC compared to those with SS and NASH without HCC

Clinical characteristics	SS ($n = 13$)	NASH		P value[a]	P value[b]
		Without HCC ($n = 30$)	HCC ($n = 6$)		
Age (range), years	51 (29–66)	53 (24–77)	73 (72–80)	0.087	<0.001
Male gender (%)	69	40	50	0.114	0.677
BMI (range), kg/m^2	27.4 (22.4–35.3)	27.1 (19.2–35.1)	25.4 (23.5–28.9)	0.659	0.358
Blood pressure (range), mmHg					
Systolic	122 (110–156)	124 (107–154)	134 (116–157)	0.714	0.136
Diastolic	80 (50–110)	74 (50–93)	72 (60–82)	0.548	0.511
Hypertension (%)	42	41	100	0.182	0.138
FBS (range), mg/dL	91 (82–101)	107 (82–233)	154 (109–212)	0.004	0.012
Fasting insulin (range), μU/mL	10.0 (6.3–23.2)	19.2 (4.2–31.6)	20.8 (4.7–21.4)	0.092	0.700
HOMA-R (range)	2.10 (1.34–5.27)	5.16 (0.94–8.60)	5.60 (2.46–8.03)	0.009	0.817
HbA1c (range), %	5.3 (5.1–5.8)	5.6 (4.4–14.5)	6.1 (5.1–8.8)	0.041	0.517
Diabetes (%)	0	40	67	0.004	0.374
Total cholesterol (range), mg/dL	213 (178–237)	194 (118–310)	176 (159–230)	0.347	0.604
Triglycerides (range), mg/dL	237 (82–536)	168 (49–867)	155 (81–288)	0.198	0.759
Uric acid (range), mg/dL	5.5 (4.1–8.3)	6.2 (2.9–8.5)	5.1 (4.3–6.4)	0.772	0.115
CRP (range), mg/dL	0.15 (0.10–0.50)	0.20 (0.10–0.85)	0.74 (0.10–1.68)	0.102	0.127
Concomitant malignancy (%)	15	17	33	>0.999	0.573
AST (range), IU/L	36 (18–75)	44 (22–113)	43 (25–51)	0.110	0.640
ALT (range), IU/L	79 (20–141)	60 (20–133)	35 (18–126)	0.448	0.270
γ-GTP (range), IU/L	72 (24–718)	94 (26–1101)	58 (49–124)	0.341	0.278
Platelet (range), $\times 10^4$/μL	22.7 (14.4–33.9)	20.5 (5.3–31.9)	22.2 (7.1–33.4)	0.258	0.497
Type IV collagen (range), ng/mL	124 (101–179)	192 (95–352)	227 (185–405)	0.007	0.228
Serum Fe (range), μg/dL	85 (40–132)	101 (28–161)	57 (53–130)	0.129	0.254
Serum ferritin (range)[c]	0.693 (0.070–1.544)	1.432 (0.092–6.480)	1.698 (0.246–3.604)	0.043	0.920
Transferrin saturation (range), %	29.3 (11.6–37.8)	31.0 (6.0–48.5)	27.6 (15.7–43.5)	0.677	0.702

HCC hepatocellular carcinoma, *NASH* nonalcoholic steatohepatitis, *SS* simple steatosis, *BMI* body mass index, *FBS* fasting blood sugar, *HOMA-R* homeostatic model assessment ratio, *HbA1c* glycated hemoglobin, *CRP* C-reactive protein, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *γ-GTP* gamma-glutamyl transpeptidase

[a] Patients with SS vs. those with NASH without HCC

[b] Patients with NASH without HCC vs. those with NASH-HCC

[c] Values divided by the upper limit of normal separately in males and females

Table 2 Pathological findings for patients with NASH–HCC compared to those for patients with SS and with NASH without HCC

Pathological findings (%)	SS (n = 13)	NASH		P value[a]	P value[b]
		Without HCC (n = 30)	HCC (n = 6)		
Steatosis[c] (0/1/2/3)	0/38/38/24	0/33/47/20	0/83/0/17	0.909	0.082
Lobular inflammation[d] (0/1/2/3)	31/62/7/0	0/37/57/6	0/50/50/0	<0.001	0.468
Ballooning[e] (0/1/2)	100/0/0	0/57/43	0/67/33	<0.001	0.655
NAS (range)	3 (1–4)	5 (3–7)	4 (4–5)	<0.001	0.059
Mallory's hyaline	0	17	0	0.301	0.564
Brunt's classification					
Grade (0/1/2/3)	69/31/0/0	3/30/60/7	0/33/67/0	<0.001	0.883
Stage (0/1/2/3/4)	100/0/0/0/0	10/43/20/10/17	17/17/17/50/0	<0.001	0.643
Iron staining					
Grade (0/1/2/3/4)	85/15/0/0/0	63/26/7/0/4	66/0/17/17/0	0.141	0.827

NAS, NAFLD activity score

[a] Patients with SS vs. those with NASH without HCC

[b] Patients with NASH without HCC vs. those with NASH–HCC

[c] Score: 0, <5 %; 1, 5–33%; 2, 34–66 %; 3, >66 %

[d] Score: 0, none; 1, <2 at 20× magnification; 2, 2–4 at 20× magnification; 3, >4 at 20× magnification

[e] Score: 0, none; 1, few, 2, many

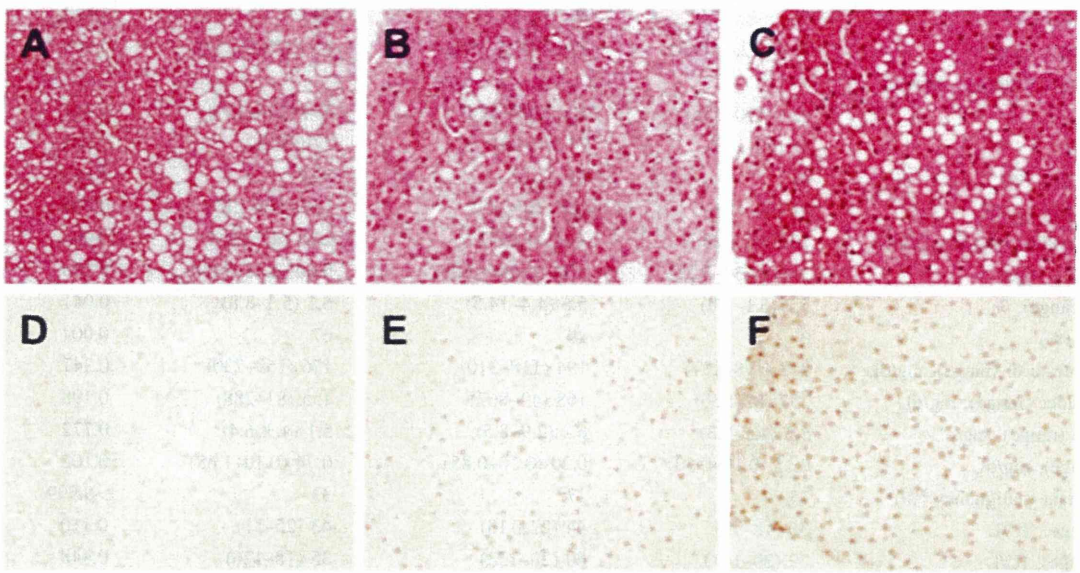

Fig. 3 Content of 8-OHdG in liver tissue from patients with nonalcoholic fatty liver disease (NAFLD). **a**, **d** Simple steatosis, **b**, **e** NASH without HCC, **c**, **f** NASH–HCC. The immunostaining intensity of **d**, **e**, and **f** was 1.015, 3.671, and 8.605, respectively. Staining: **a–c** hematoxylin and eosin, **d–f** 8-OHdG immunostaining. Magnification: ×200 (**a–f**)

patients with NASH without HCC than in those with SS (P = 0.020), but there was no significant difference between patients with NASH–HCC and those with NASH without HCC (P = 0.423) (Fig. 5).

Multivariate analysis carried out using hepatic 8-OHdG content as a factor in addition to age and FBS showed a significant difference in clinicopathological factors between NASH–HCC cases and cases of NASH without HCC. Old age [P = 0.015; odds ratio (OR) 1.366; 95 % confidence interval (CI) 1.038–2.524] and high relative immunohistochemical intensity for intrahepatic 8-OHdG (P = 0.037; OR 2.725; 95 % CI 1.050–21.903) were identified as independent factors (Table 3). The correlation between intrahepatic 8-OHdG content and background factors was analyzed for NAFLD cases and the intrahepatic 8-OHdG content correlated significantly with FBS, homeostasis

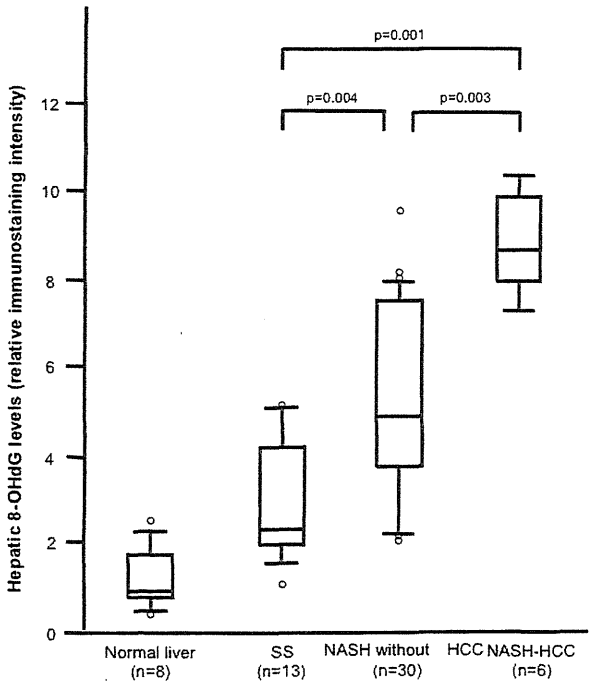

Fig. 4 Comparison between hepatic 8-OHdG levels in patients with simple steatosis (*SS*), NASH without HCC, and NASH–HCC (non-cancerous tissue). Relative staining intensity of hepatic 8-OHdG was quantified by computer-assisted image analysis. The results are shown as *box plot profiles*, with the *bottom* and *top edges* of the *boxes* representing the 25th and 75th percentiles, respectively. Median values are shown by the *line within the box*. Values outside the top or bottom 95th percentiles are shown individually. *P* values for comparison of normal liver with SS, NASH without HCC, and NASH–HCC were 0.002, <0.001, and 0.003, respectively

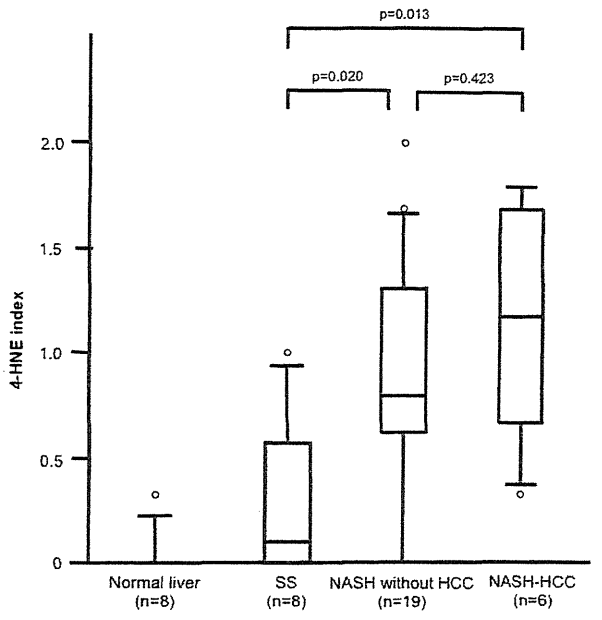

Fig. 5 Semi-quantitative assessment of 4-hydroxy-2-nonenal (*HNE*) immunostaining in patients with NASH–HCC compared to those with SS and with NASH without HCC (non-cancerous tissue). The results are shown as *box plot profiles*, with the *bottom* and *top edges* of the *boxes* representing the 25th and 75th percentiles, respectively. Median values are shown by the *line within the box*. Values outside the top or bottom 95th percentiles are shown individually. *P* values for comparison of normal liver with that of patients with SS, NASH without HCC, and NASH–HCC were 0.110, <0.001, and 0.001, respectively

model assessment ratio (HOMA-R), CRP, serum ferritin (adjusted values), histological grades and stages, and liver iron staining grade (Table 4).

Table 3 Multivariate analysis of factors associated with hepatocarcinogenesis in patients with NASH

Factors	Odds ratio (95 % CI)	*P* value
Age	1.366 (1.038–2.524)	0.015
FBS	1.033 (0.974–1.127)	0.257
Hepatic 8-OHdG	2.725 (1.050–21.903)	0.037

CI confidence interval

Discussion

It has been demonstrated that NASH is precipitated by inflammation and fibrosis when a variety of hepatocyte-damaging factors, such as insulin resistance, mitochondrial dysfunction, adipocytokines, endotoxin, inflammatory cytokines, and excessive iron deposition, occur along with the formation of fatty liver in patients with a background of lipid metabolic disorder and obesity [10–12, 28–31]. These factors are known to accelerate oxidative stress. Based on the observation of an increased production of reactive oxygen species (ROS) in hepatocyte mitochondria and hepatocyte hyperplasia in an insulin-resistant obese mouse model [10, 11], it has been speculated that ROS production, which increases during the progression of NASH, contributes to not only inflammation but also carcinogenesis. A number of studies have examined the influence of oxidative stress on the onset and development of HCC in humans in terms of the association of oxidative stress with poor histological differentiation and tumor size [32], increased neovascularization by oxidative stress [33], and activation of telomerase by oxidative stress [34]. In these studies, the 8-OHdG content was measured histologically as a marker of oxidative stress, and all studies showed that the 8-OHdG content was greater in HCC tissues than in non-cancerous tissue. However, the distribution of background liver diseases varied in these studies (e.g. HBV infection, HCV infection, ALD, etc.).

We previously reported that intrahepatic accumulation of 8-OHdG was related to hepatocarcinogenesis in patients

Table 4 Correlations between clinical findings and 8-OHdG levels in the liver of patients with NAFLD

Clinical parameters	Statistics	
	r	*P* value
Age (years)	0.232	0.109
BMI (kg/m^2)	0.002	0.991
Blood pressure (mmHg)		
Systolic	0.097	0.518
Diastolic	0.008	0.957
FBS (mg/dL)	0.348	0.017
Fasting insulin (μU/mL)	0.282	0.107
HOMA-R	0.365	0.031
HbA1c (%)	0.061	0.725
Total cholesterol (mg/dL)	0.075	0.615
Triglycerides (mg/dL)	−0.019	0.899
Uric acid (mg/dL)	−0.171	0.246
CRP (mg/dL)	0.460	0.002
AST (IU/L)	0.250	0.083
ALT (IU/L)	0.131	0.372
γ-GTP (IU/L)	−0.022	0.883
Platelet (×10^4/μL)	0.024	0.874
Type IV collagen (ng/mL)	0.356	0.063
Serum Fe (μg/dL)	0.057	0.716
Serum ferritin[a]	0.317	0.033
Transferrin saturation (%)	0.030	0.876
Iron staining	0.348	0.017
Brunt's classification		
Grade	0.454	<0.001
Stage	0.360	0.011

NAFLD nonalcoholic fatty liver disease

[a] Values divided by the upper limit of normal separately in males and females

with chronic hepatitis C [16], to our knowledge, our study is the first to investigate the 8-OHdG content in HCC tissue from patients with different background diseases, including NASH. We compared NASH–HCC cases with HBV–HCC and ALD–HCC cases and found that the 8-OHdG content measured immunohistochemically in cancerous and non-cancerous tissues was significantly increased in NASH–HCC patients and comparable to that in patients with HCV–HCC. In our previous study [16], two independent methods, electrochemical detection and semiquantitative immunohistochemical analysis, were used for measuring 8-OHdG levels in liver specimens. Values obtained by both methods showed a reasonable correlation, indicating the equal validity of both methods. Based on these results, we considered that the reactive staining intensity to be correlated with the absolute amount of 8-OHdG. However, in terms of practicality, the availability of formalin-fixed samples made immunostaining less laborious than the former method, which requires the use of freshly frozen biopsy samples. Consequently, we only used immunostaining in the present study. The results suggest that the accumulation of 8-OHdG in the liver is a risk factor for carcinogenesis in NASH patients, as it is in HCV-infected patients.

The intrahepatic 8-OHdG content in NASH–HCC patients was then compared with that in patients with SS and in those with NASH without HCC. As liver disease advanced, the 8-OHdG content significantly increased in a step-by-step manner. Seki et al. [27] reported that the intrahepatic 8-OHdG content increased relative to SS and healthy subjects and was associated with inflammation; however, we found that hepatic 8-OHdG content was related not only to an increase in the inflammation score but also to carcinogenesis.

Only a few studies have examined the risk factors for hepatocarcinogenesis in NASH cases. Ascha et al. [4] reported that old age at the time of diagnosis of liver cirrhosis and low-level alcohol consumption were risk factors for carcinogenesis; however, the subjects in their study were patients on a waiting list for liver transplantation, and the authors presumed that liver cirrhosis was advanced in these patients. Kawamura et al. [7] reported that age ≥60 years, AST level of ≥40 IU/L, platelet count of ≤150,000/μL, diabetes, and AST to platelet ratio index (AST level/platelet count × 100) of >1.5 were characteristic of liver steatosis patients, as diagnosed by imaging modalities, who developed HCC. Hashimoto et al. [8] demonstrated that old age, low AST, low hepatitis activity, and liver fibrosis at stage F3–F4 versus F1–F2 were risk factors for hepatocarcinogenesis.

In contrast, the Japan NASH Study Group performed a cross-sectional study that characterized the clinical features of NASH patients who developed HCC and detected high rates of obesity, type 2 diabetes, and hypertension among all patients. Furthermore, HCC appears to develop at a less advanced stage of liver fibrosis in male versus female patients [6]. Although the risk factors for hepatocarcinogenesis remain controversial, old age and impaired glucose tolerance seem to be commonly suggested.

In our study, when background factors were compared in patients with NASH–HCC and NASH without HCC, NASH–HCC patients were significantly older and had higher FBS, which is consistent with previously reported data. Multivariate analysis of these factors and 8-OHdG content in the liver tissue revealed that old age and 8-OHdG content were independent risk factors for hepatocarcinogenesis. Therefore, these factors relating to 8-OHdG content in the liver tissue of NAFLD patients were explored further.

Fujita et al. [35] reported that the 8-OHdG content in the liver tissue of NASH patients increased compared with that

of SS patients and that this increase was related to iron accumulation and insulin resistance. These authors also found that the 8-OHdG content was decreased by phlebotomy in NASH patients with iron overload. In this study [35] the authors performed correlational analysis separately on SS and NASH cases and found that the factors significantly correlated with the 8-OHdG content—only in SS cases—were age and serum ferritin levels and that there was no correlation with insulin resistance. However, since the SS group included some patients who would likely develop NASH in the future and the NASH without HCC group included some patients who would likely develop HCC, the 8-OHdG content and other factors were subjected to correlational analysis for all NAFLD cases including both SS and NASH cases. As a result, the intrahepatic 8-OHdG content showed a significant correlation with FBS, HOMA-R, CRP, serum ferritin (adjusted values), histological grades and stages, and liver iron staining grade. It has been suggested that 8-OHdG content in liver tissue serves a marker of oxidative stress triggered by a variety of factors, such as impaired glucose tolerance, inflammation, fibrosis, and iron overload.

In conclusion, oxidative DNA damage affects hepatocarcinogenesis in patients with NASH, and 8-OHdG content in liver tissue is particularly useful for predicting hepatocarcinogenesis. However, a future prospective follow-up is warranted for validation of these results.

Conflict of interest None.

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