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1 血液検査

非アルコール性脂肪肝炎 nonalcoholic steatohepatitis（NASH）の診断のための血液検査のひとつは，NASHの基盤となる非アルコール性脂肪性肝疾患 non-alcoholic fatty liver disease（NAFLD）の診断における血液検査である．これには，脂肪肝に特徴的な血液検査に加えて，NASHへの病態の進展を評価する血液検査が含まれる．また，NASHの診断には，アルコール性脂肪肝だけでなく，各種肝炎ウイルス，自己免疫，先天的肝代謝異常など他の原因による肝疾患を除外する必要がある．したがって，除外診断のための血液検査も必要である．さらにNAFLDやNASHの確定診断には肝組織所見が不可欠であるが，NAFLDの頻度は高く，肝生検は侵襲的であるため，血液検査は，肝生検を行う患者を選択するためにも重要である．

スクリーニング検査でNAFLDが考えられる症例のうち，特に以下のような血液検査所見から，単純性脂肪肝よりNASHを疑い（表5-1），肝生検による確定診断を行う．さらに，NASHは，Dayらが提唱したtwo hit theoryによると，肥満，高脂血症，糖尿病などの1st hitによる脂肪肝に，インスリン抵抗性や酸化ストレス，エンドトキシンなどの2nd hitが加わって発症する．したがって，このtwo hit theoryに関連する因子に関する血液検査は，NAFLDやNASHの診断にも有用であると考えられる．

表5-1　肝生検を考慮すべきNAFLD患者の血液検査所見

1．血液・生化学検査
- トランスアミナーゼの持続高値（特にALT100以上が持続）
- AST/ALT比の経時的上昇
- コリンエステラーゼの経時的低下
- 肝線維化マーカー（ヒアルロン酸，Ⅳ型コラーゲン，PⅢP）高値
- 血小板数の低下
- 血糖コントロール不良
- 血清フェリチン高値
- HOMA-IRの上昇（2以上）

2．特殊検査
- 高感度CRP高値
- 血清チオレドキシン高値
- TNF-α高値
- アディポネクチン低値
- レプチン高値
- 血清CK18断片高値
- DHEA-S高値

A. NAFLD および NASH 診断に有用な血液生化学検査

AST, ALT の軽度上昇が NAFLD ではみられ，さらに NASH では高い傾向にある（表 5-2）[1]．しかし正常の 5 倍以上になることはほとんどなく，NASH の 2/3 は正常値まで変動することがある[2]．NASH は ALT 優位で AST/ALT 比は 1 未満であり，1 以上となるアルコール性脂肪肝などと鑑別される．さらに，コリンエステラーゼも NAFLD や NASH で上昇する．しかし，ウイルス性慢性肝疾患と同様に NASH でも AST/ALT 比は肝線維化の進行とともに上昇し，肝硬変では 1 以上になることから，経過観察には有用なマーカーの一つである．コリンエステラーゼもアルブミンやプロトロンビン時間と同様に肝硬変に進展すると低下する．また，NAFLD のなかで肝線維化進展例は肝線維化非進展例と比較してトランスアミナーゼは高値であることも報告されており[3]，トランスアミナーゼが持続高値であれば，NASH を疑う根拠となる．ALP，γGTP は NAFLD で上昇することがあるが，軽度であり，NASH と単純性脂肪肝の鑑別には有用ではない．

肝線維化マーカーであるヒアルロン酸，Ⅳ型コラーゲン，procollagen Ⅲ polypeptide（PⅢP）は単純性脂肪肝より NASH で高値となると考えられ，血小板数は低値となる．また，肝合成能の指標であるアルブミン，プロトロンビン時間は NASH の病態進展とともに低下するが，軽度の肝線維化を呈する NASH では単純性脂肪肝とほとんど変わらない．

耐糖能異常，脂質代謝異常，高血圧などの生活習慣病は NASH の発症や病態進展と関連することから，血糖，HbA_{1c}，総コレステロール，LDL コレステロール，中性脂肪などは高値となることが多い．また，NASH は単純性脂肪肝と比較して肝組織中の鉄沈着の程度が高度であり，血清フェリチンは NASH では高値となる．高フェリチン血症と肝組織炎症や線維化重症度との関連もあるといわれる[4]．細胞内で過剰となった鉄がフリー鉄となり，Fenton 反応により ROS の産生を亢進させ，肝細胞障害や肝線維化を惹起すると考えられている．

表 5-2　NASH と単純性脂肪肝患者の生化学検査値の比較*

	steatosis patients	NASH patients	*P* value
AST（U/m*l*）	33.3 ± 12.8	53.0 ± 35.5	0.01
ALT（U/m*l*）	57.0 ± 32.3	80.6 ± 60.0	0.08
FBS（m*l*/m*l*）	108.4 ± 16.4	123.6 ± 36.8	0.06
IRI（μ*l*/m*l*）	11.4 ± 7.8	14.1 ± 10.6	0.28
HOMA-IR	3.10 ± 2.30	4.07 ± 3.73	0.26
HDL cholesterol（mg/d*l*）	49.9 ± 14.4	47.9 ± 11.5	0.50
LDL cholesterol（mg/d*l*）	115.1 ± 31.0	129.3 ± 38.5	0.13
triglycerides（mg/d*l*）	170.7 ± 82.3	172.6 ± 98.5	0.94
iron（ng/m*l*）	116.1 ± 46.9	110.1 ± 40.2	0.59
ferritin（ng/m*l*）	146.6 ± 96.1	264.2 ± 245.4	0.05
hyaluronic acid（ng/d*l*）	27.3 ± 26.2	51.2 ± 52.2	0.05
type Ⅳ col.7s（ng/d*l*）	4.21 ± 0.93	4.67 ± 1.23	0.12

（* means ± SD．文献 1 を改変）

B. 他の肝疾患除外のための血液検査

まず，ウイルス性肝炎の除外のためにHBs抗原とHCV抗体を測定し，自己免疫性肝疾患の可能性を否定するために，抗ミトコンドリア抗体と抗核抗体を測定する．ただし，NAFLDでは抗核抗体陽性者が存在することから，検査結果の解釈には注意が必要である．特徴的な臨床経過や症状などから，先天的肝代謝異常が疑われる場合には，セルロプラスミン，尿中銅，α_1アンチトリプシン，トランスフェリン飽和度などそれらに特異的な検査を行う（図5-1）．

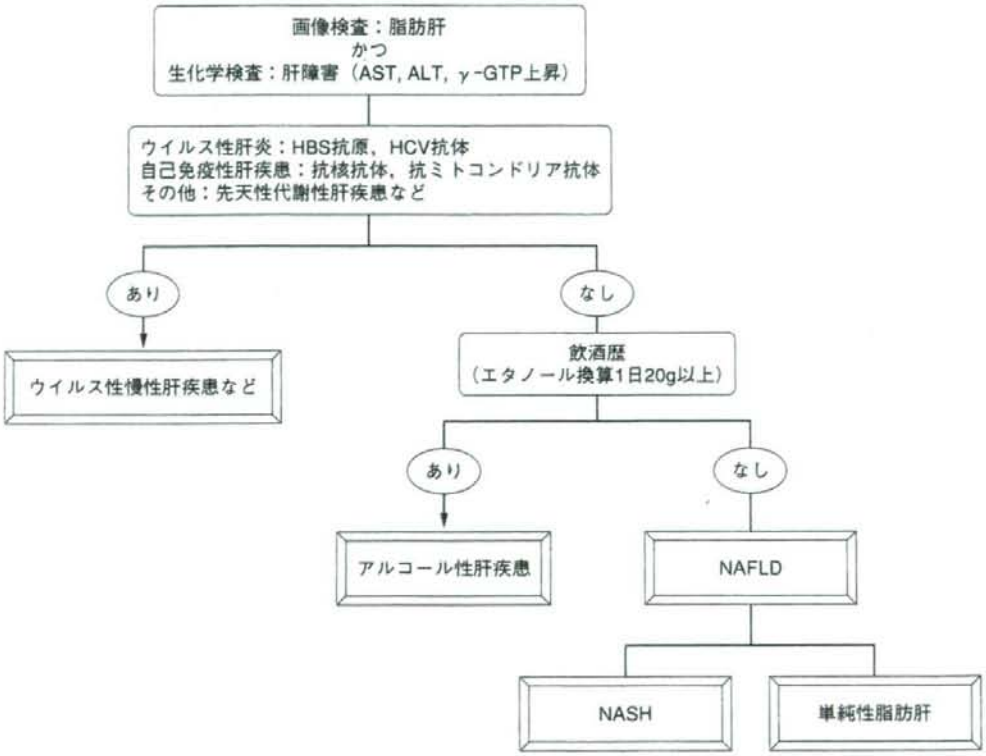

図5-1 NASH/NAFLDの診断

C. NAFLDおよびNASHの病態に関連する血液検査

1. インスリン抵抗性

内臓脂肪蓄積はNAFLDの基盤として重要であり，それに伴うインスリン抵抗性はNASHの基盤となる病態である．インスリン抵抗性の評価法はいくつかあるが，現在最も応用されているのは，HOMA-IR（homeostasis model assessment insulin resistance index）で，1回の採血で評価できることから汎用されている．HOMA-IRは空腹時血糖（mg/dl）×空腹時インスリン（μU/ml）÷405の計算式で求められる．一般に，HOMA-IRが2未満の場合は正常，2以上はイ

ンスリン抵抗性．5以上の場合は高度のインスリン抵抗性と考えられる．脂肪肝の程度が進行するほどHOMA-IRが高値となり，血清ALT値はBMI，HOMA-IRと有意に正の相関を示し，ALTが40U/*l*以上の例の大半がBMIが25以上，あるいはHOMA-IRが2以上である[5]．また，NASHの肝線維化の進行はインスリン抵抗性と関連があるといわれている[3]．このようなことから，インスリン抵抗性の存在はNASHを疑う所見の一つである．

2. 高感度CRP（high-sensitivity C-reactive protein: hsCRP）

高感度CRP測定法の開発により，従来のCRPでは検出されない慢性的で微小な炎症を評価できるようになった．hsCRPレベルの上昇は，心血管疾患，2型糖尿病，メタボリックシンドローム発症の予測因子であることが証明されている[6]．また，メタボリックシンドロームの一表現形といわれるNAFLDではhsCRPの上昇が脂肪肝に伴う炎症を反映していると考えられている．さらにNASHにおいてhsCRPの上昇と肝線維化の程度には相関があるといわれ[1]，メタボリックシンドロームを有しないNAFLDの血清ALT高値群と正常群の比較では，有意にALT高値群のhsCRPが高値であるという報告[5]もあり，今後NASHの新しいマーカーとなることが期待される（図5-2）．

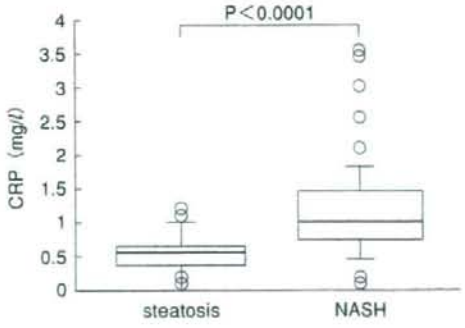

図5-2　NASHと単純性脂肪肝におけるhsCRP濃度（文献1を改変）

3. 酸化ストレスマーカー

ミトコンドリアの呼吸鎖複合体はROSを産生し，通常は速やかに消去されるが，NASHではミトコンドリア異常によりROS過剰になると考えられている．また，肝細胞の過剰な脂肪酸はミトコンドリアのβ酸化を亢進させ，CYP2E1の過剰発現を誘導する．さらに，NASH患者の肝組織中の鉄沈着レベルは単純性脂肪肝より高値である．このような病態はNASHにおいて酸化ストレスを誘導すると考えられる．

酸化ストレスマーカーには，活性酸素による修飾化合物と活性酸素消去系酵素や抗酸化物質がある[7]．heat shock protein（HSP）やチオレドキシン thioredoxin（TRX）などは酸化ストレスによって誘導される蛋白質であり，チオレドキシンは抗酸化物質としても知られている．NAFLDでは健常者と比較して，肝組織中のmalondialdehyde（MDA）など酸化ストレスマーカーは発現が高く，NAFLDの中でも単純性脂肪肝よりNASHのほうが，肝組織中MDAや血

清中チオレドキシンが高い（図5-3）[8]．このように，NASHの病態形成に酸化ストレスが強く関与すると考えられ，酸化ストレスマーカーはNASHの診断や病態進展予測に役立つ．特に，チオレドキシンは血清中の濃度を測定できることから，診断マーカーとしての有用性が期待できる．

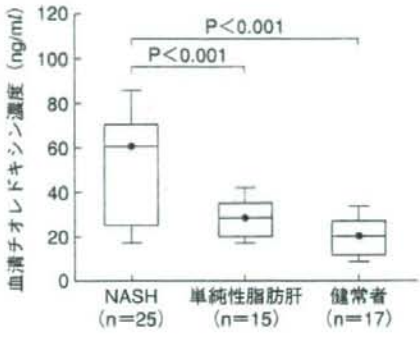

図5-3　**NASH，単純性脂肪肝と健常者における血清チオレドキシン濃度**（文献8を改変）

4. アディポサイトカイン

脂肪組織は単なる余剰エネルギーの蓄積臓器であるだけでなく，非常に多くの生理活性物質を分泌する組織である．このような脂肪組織由来生理活性物質は総称してアディポサイトカインとよばれる．代表的なアディポサイトカインとして，TNF-α，レプチン，アディポネクチン，PAI-1（plasminogen activator inhibitor type1），レジスチンなどがあげられるが，NAFLDの中でも特にNASHではこれらの分泌異常があるといわれる．

TNF-αは炎症性サイトカインの一つであるが，脂肪組織においても発現している．肥満により血中TNF-αは上昇し，脂肪組織におけるTNF-αの発現は肥満度と相関し，NASHでは血中TNF-αの上昇や肝でのTNF-α受容体の増加が認められる[9,10]．

アディポネクチンはメタボリックシンドローム発症に抑制的に働き，肥満によりその血中濃度は低下し，内臓脂肪量と強い逆相関を示す．骨格筋や肝臓においてAMP kinaseやPPARαを活性化し，インスリン抵抗性やメタボリックシンドロームを改善する作用をもつ．NAFLD患者では血中アディポネクチン濃度は低下している[11]．また，インスリン抵抗性改善薬ピオグリタゾンは，PPARγを介して脂肪細胞を小型化し，アディポネクチン産生低下を回復することで

MEMO

プロテオミクス

プロテオーム（proteome＝protein＋ome）とは1個の生物がもつすべての遺伝情報であるゲノム（gene＋ome）に相応した用語であり，その生物や細胞に発現しているすべての蛋白質の集合体のことである．また，網羅的に蛋白質の発現や性質を研究することを，プロテオーム解析もしくはプロテオミクス proteomicsと表現する．プロテオーム解析は(1)蛋白質の分離，(2)蛋白質の同定の2つの作業からなる．最も広く行われているのは，二次元電気泳動によって蛋白質を分離し，質量分析で目的の蛋白質を同定する方法である．質量分析には液体クロマトグラフィーと一体となったLC-MSと蛋白質の質量と飛行時間の関係を利用したTOF-MSがある．

NASH 患者に有効であると考えられている．このようなことから，アディポネクチンは診断だけでなく，アディポネクチンを用いた治療やピオグリタゾンなどの治療薬の効果のモニタリングにも応用できる可能性がある．

レプチンは中枢神経系に作用して強力な食欲抑制作用を示すほか，インスリン効果増強作用，エネルギー消費亢進，血圧上昇作用などさまざまな生理作用を示す．体脂肪量が増加するとレプチン濃度が上昇し，食欲を低下させるとともにエネルギーの消費を高めるが，肥満患者ではレプチン抵抗性の状態と考えられ，その機構が破綻した状況となっている．また，レプチンは肝線維化と関連する肝星細胞の活性化を促進することが報告されている[9,12]．このように，血中レプチン濃度は NASH では上昇し，診断に有用である可能性がある．

D. 血液検査の next approach

NAFLD や NASH のスクリーニングのための血液検査は簡便に測定できる．しかし，NASH を疑う根拠となるような検査は保険外診療で測定しなければならない検査項目も多く，必ずしも NAFLD もしくは NASH に特異的なものではなく，NASH の確定診断には肝生検が必要である．すなわち，簡便に測定でき，臨床の場で有用な NASH の特異的な血清診断マーカーはない．

血清 cytokeratin-18（CK-18）断片濃度は NAFLD における肝細胞のアポトーシスと関連して増加し[13]，男性ホルモンの中間代謝産物であるデヒドロエピアンドロステロンサルフェート（DHEA-S）は NAFLD の肝線維化の進行とともに低下する[14]．さらに，いくつかのマーカーを組み合わせた早期 NASH の診断法も報告されるようになってきている（表 5-3）[15]．また，プロ

表 5-3　アディポネクチン，HOMA-IR，Ⅳ型コラーゲン 7S，およびマーカーの組み合わせを用いた線維化軽度（Stage1-2）NASH の診断率

parameter	cut-off Value	AUC	感度	特異度
adiponectin	≦4.0（μg/ml）	0.765	68	79
HOMA-IR	≧3.0	0.757	51	95
Type Ⅳ Col.7S	≦5.0（ng/ml）	0.758	41	95
combination of markers			94	74

（文献 15 を改変）

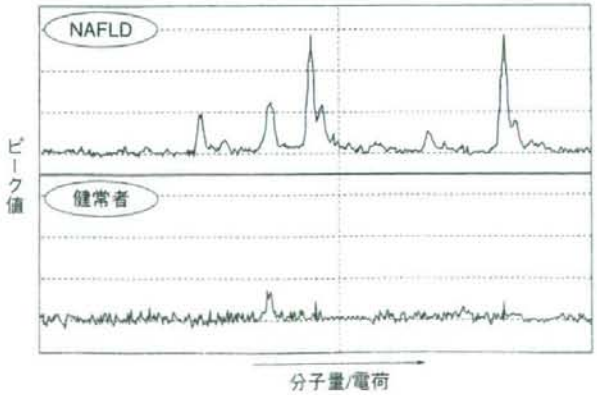

図 5-4　SELDI-TOF/MS プロテインチップシステムを用いた NAFLD 患者血清の蛋白発現パターン

テオミクスなどを用いたバイオマーカー探索が悪性疾患を中心に広く行われるようになってきているが，NAFLDやNASHにも応用可能あると考えられ[16,17]，今後新しいバイオマーカーの発見が期待されている．我々も，プロテオミクスを用いて，健常者に比較してNAFLDで高値を示し，NAFLDのバイオマーカーとなる可能性がある蛋白ピークをみいだしており（図5-4），今後の研究の発展が期待できる．

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Hepatic oxidative DNA damage is associated with increased risk for hepatocellular carcinoma in chronic hepatitis C

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Although the oxidative stress frequently occurs in patients with chronic hepatitis C, its role in future hepatocellular carcinoma (HCC) development is unknown. Hepatic 8-hydroxydeoxyguanosine (8-OHdG) was quantified using liver biopsy samples from 118 naïve patients who underwent liver biopsy from 1995 to 2001. The predictability of 8-OHdG for future HCC development and its relations to epidemiologic, biochemical and histological baseline characteristics were evaluated. During the follow-up period (mean was 6.7 ± 3.3 years), HCC was identified in 36 patients (30.5%). Univariate analysis revealed that 16 variables, including 8-OHdG counts (65.2 ± 20.2 *vs* 40.0 ± 23.5 cells per 10^5 μm^2, $P<0.0001$), were significantly different between patients with and without HCC. Cox proportional hazard analysis showed that the hepatic 8-OHdG ($P=0.0058$) and fibrosis ($P=0.0181$) were independent predicting factors of HCC. Remarkably, 8-OHdG levels were positively correlated with body and hepatic iron storage markers (*vs* ferritin, $P<0.0001$ *vs* hepatic iron score, $P<0.0001$). This study showed that oxidative DNA damage is associated with increased risk for HCC and hepatic 8-OHdG levels are useful as markers to identify the extreme high-risk subgroup. The strong correlation between hepatic DNA damage and iron overload suggests that the iron content may be a strong mediator of oxidative stress and iron reduction may reduce HCC incidence in patients with chronic hepatitis C.

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Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide, and the death rate due to this tumour has been increasing over the past 20–30 years in the Unites States (El-Serag, 2002) and in Japan (Nishioka *et al*, 1991). Chronic infection with hepatitis C virus (HCV) has been recognised as an increased risk of HCC; approximately 20% of HCV-infected individuals have diseases that progress to cirrhosis, and about 40% of these patients develop HCC after a mean of 10–15 years (Seeff, 2002). Consequently, surveillance programmes based on periodic ultrasound examination and serum α-fetoprotein determination are recommended for patients with chronic hepatitis C. However, the effectiveness of these protocols has not been fully assessed and they afford no contribution to improvement of clinical outcomes in patients with chronic hepatitis C (Gebo *et al*, 2002). Therefore, it is desirable to establish a useful marker that could identify cases at high risk of developing HCC among chronic HCV-infected patients.

Although the mechanisms underlying HCC development during chronic HCV infection have been widely investigated, they are still unclear. It has been reported that structural and nonstructural proteins of HCV, such as Core and NS3, play a role in cell transformation, using *in vitro* cell culture systems (Sakamuro *et al*, 1995; Ray *et al*, 1996) and transgenic animals (Moriya *et al*, 1998). But whether expression of HCV protein(s) directly induces HCC in chronic HCV-infected patients is unknown. Recently, it has been assumed that oxidative stress may be relevant to this process of HCV-induced carcinogenesis, as has been suggested in several other malignancies (Kasai, 1997). Considerable data suggest that reactive oxygen species (ROS) may play a pathogenic role in carcinogenesis (Crawford and Cerutti, 1985). The most damaging species among the many ROS is the hydroxyl radical. The hydroxyl radical has been shown to be responsible for a number of base modifications that include thymine glycol, thymidine glycol (Cathcart *et al*, 1984), 5-(hydroxylmethyl)uracil (Hollstein *et al*, 1984), and also 8-hydroxydeoxyguanosine (8-OHdG) (Shigenaga *et al*, 1989; Kasai, 1997). 8-Hydroxydeoxyguanosine is a modification of guanine that induces a point mutation in the daughter DNA strands (Kuchino *et al*, 1987; Shibutani *et al*, 1991) and it is therefore used as a marker of oxidatively generated DNA damage in several diseases (Shigenaga *et al*, 1989; Kasai, 1997). In patients with chronic hepatitis C, increased 8-OHdG in DNA extracted from liver tissue was also reported (Shimoda *et al*, 1994; Mahmood *et al*, 2004; Fujita *et al*, 2007). These reports suggest that oxidative stress may be involved in the progression of liver disease, but they showed no direct participation of oxidative stress in hepato-

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carcinogenesis in the liver of HCV-infected patients. Also, no information is available on whether ROS-mediated damage to DNA is useful for prediction of HCC development in chronic hepatitis C patients.

In view of these considerations, we have examined the influence of the degree of ROS-mediated hepatic DNA damage as measured by counts of 8-OHdG immunohistochemically positive hepatocyte nuclei on the prevalence of future HCC development in chronic HCV-infected patients. Moreover, we evaluated the relation of the degree of ROS-mediated DNA damage with the epidemiologic, biochemical, and histological findings in chronic hepatitis C.

PATIENTS AND METHODS

Patients with chronic hepatitis C

This study comprised 118 consecutive patients (66 males and 52 females; mean age 55.8 ± 10.8 years) recruited between January 1995 and October 2001 with HCV-related chronic hepatitis (Table 1). All patients fulfilled the following inclusion criteria: (1) liver injury caused by chronic HCV infection. All patients had persistently elevated serum alanine aminotransferase (ALT) levels and were seropositive for both anti-HCV antibody (the third-generation enzyme-linked immunosorbent assay; Ortho Diagnostic Systems, Raritan, NJ, USA) and HCV-RNA (Amplicor-HCV assay; Roche Molecular Diag. Co., Tokyo, Japan). (2) Liver biopsy. Liver tissue was obtained by percutaneous needle biopsy in all patients for diagnostic purposes. (3) Follow-up without interferon (IFN)-based therapy. The follow-up consisted of monthly blood tests and monitoring of tumour markers at the outpatients clinic of our department, and ultrasonography and dynamic computed tomography were performed at regular intervals. As it is known that IFN treatment reduces the incidence of HCC in patients with chronic hepatitis C (Nishiguchi *et al*, 1995), patients with a history of previous IFN-based treatment were excluded from the study. None of them had received any antiviral therapy during the follow-up period.

Exclusion criteria were as follows: a family history of haemochromatosis; haemolytic disease; serological markers for hepatitis B virus (HBV) (hepatitis B surface antigen and hepatitis B core antibody); or human immunodeficiency virus infection. Patients with concurrent diseases or those taking medications that may interfere with free radical production, such as nonsteroidal anti-inflammatory drugs, vitamins and iron-containing drugs, were excluded from the study. Patients with chronic alcohol consumption of ethanol in excess of 40 g day^{-1} for male and 20 g day^{-1} for female for at least 5 years were also excluded. All patients had no HCC or other cancers, by an initial screening examination. Informed consent was obtained from each patient and the study was approved by the Ethical Committee of Mie University. The study was carried out according to the ethical guidelines of the 1975 Declaration of Helsinki.

Clinical parameters were obtained for each patient at the time of liver biopsy: age; sex; body mass index; duration of HCV infection (when contamination was very probable and a precise data; transfusion or drug addiction in the past year); alcohol intake; biochemical, haematological, iron-related, and virological serum markers; and liver histological findings and 8-OHdG immunoreactivity.

The diagnosis of HCC was made by several imaging methods (ultrasonography, dynamic computed tomography, arteriography, or magnetic resonance imaging) and confirmed histologically in 22 cases. Time to HCC occurrence was defined as the interval between the date of liver biopsy and the detection of tumour, death without HCC occurrence, or the last examination until 31 October 2006. All patient deaths were considered end points irrespective of cause of death. The mean follow-up period was 6.7 ± 3.3 (range, 0.4–11.8) years.

Table 1 Baseline characteristics of patients with chronic hepatitis C

Characteristics	Chronic hepatitis C ($N = 118$)
Age (years)	55.8 ± 10.8 (57.5)
Gender (M/F)	66/52
Laboratory data	
ALT (IU l^{-1})	73.5 ± 53.4 (58.0)
AST (IU l^{-1})	70.1 ± 41.8 (61.5)
Platelet count ($\times 10^4$ mm^{-3})	14.9 ± 5.9 (14.6)
Serum HCV-RNA (kIU ml^{-1}) ($N = 89$)	1570 ± 1240 (1420)
HCV genotype (1a/1b/2a/2b) ($N = 60$)	0/53/5/2
Liver histology	
Inflammatory activity (0/1/2/3)[a]	1/41/49/27
Fibrosis staging (0/1/2/3/4)[b]	1/29/26/27/35
Total iron score[c]	7.75 ± 5.80 (7.00)
8-OHdG-positive hepatocytes (per 10^3 μm^2)	48.4 ± 26.2 (42.5)

Data are expressed as mean ± s.d. (median). ALT = alanine aminotransferase; AST = aspartate aminotransferase; HCV = hepatitis C virus; 8-OHdG = 8-hydroxydeoxyguanosine. [a]Inflammatory activity was graded according to the intensity of necroinflammatory lesions: 0, no histological activity; 1, mild activity; 2, moderate activity; 3, severe activity. [b]Fibrosis staging was scored as follows: 0, no fibrosis; 1, portal fibrosis without septa; 2, portal fibrosis with few septa; 3, numerous septa without cirrhosis; 4, cirrhosis. [c]The histological quantification of iron was assessed by total iron score proposed by Deugnier *et al* (1992).

Histological evaluation

All of the liver biopsy samples were stained with haematoxylin–eosin and Masson's trichrome, and were graded for the degree of necroinflammatory activity and staged for the extent of fibrosis using the criteria of Desmet *et al* (1994). The histological quantification of hepatic iron was carried out according to Deugnier *et al* (1992) by scoring iron separately within hepatocytes (hepatic iron score, 0–36), sinusoidal cells (sinusoidal iron score, 0–12), and portal tracts or fibrotic tissue (portal iron score, 0–12) using liver samples stained with Perls' Prussian blue. The total iron score (TIS, 0–60) was defined by the sum of these scores. This score has been shown to highly correlate with the biochemical hepatic iron index and hepatic iron concentration as measured by the atomic absorption spectrophotometry in patients with chronic liver diseases (Piperno *et al*, 1998; Silvia *et al*, 2005).

Immunohistochemical study

Immunohistochemical staining of 8-OHdG was performed as previously described (Fujita *et al*, 2007). Mouse monoclonal antibody against 8-OHdG (Japanese Aging Control Institute, Shizuoka, Japan) and Alexa 488-labelled goat antibody against mouse IgG (Molecular Probes, Eugene, OR, USA) were used. The degree of immunoreactivity was estimated by counting the number of stained hepatocyte nuclei using Adobe Photoshop version 5.5 and NIH Image free software (Vers. 1.62, National Institute of Health, image program).

Statistical analysis

Results were expressed as mean ± s.d. or median. Comparisons between groups were performed using the Mann–Whitney *U*-test or Kruskal–Wallis test for continuous variables and the χ^2 or Fisher's exact test for categorical data. Correlation coefficients between numerical variables were calculated as Spearman's rank test. Cumulative HCC incidence curves were determined using the Kaplan–Meier method and the differences between groups were

assessed with the log-rank test. Cox proportional hazard regression analysis was used to identify significant factors that influence future HCC development. All tests were two-tailed, and *P*-values less than 0.05 were considered as statistically significant. Statistical analyses were performed using the SPSS 11.5 software (SPSS Inc., Chicago, IL, USA).

RESULTS

In situ detection of 8-OHdG-positive hepatocytes using biopsy samples

In the liver of patients with chronic hepatitis C, 8-OHdG immunoreactivity was strongly observed in the nuclei (weakly in the cytoplasm) of hepatocytes, Kupffer cells, and infiltrated lymphocytes (Figure 1A). The hepatocyte nuclei were differentiated from the nuclei of other cells using computed analyses at the point of nuclear shape and size. The number of 8-OHdG-positive hepatocytes in patients with chronic hepatitis C was counted from 7 to 123 cells per $10^5\,\mu m^2$, the median being 42.5 cells per $10^5\,\mu m^2$. Using the liver samples of patients with simple fatty liver as controls, immunoreactivity of 8-OHdG was faintly observed in the nuclei of hepatocytes in this experimental setting (Figure 1B). The specificity of the anti-8-OHdG antibody used in this study was confirmed by several parallel experiments. Sections in which the primary antibody was omitted or those treated with normal control serum instead of the primary 8-OHdG antibody consistently yielded negative staining. Localisation of 8-OHdG was considered specific because the recognition of hepatocytes was completely blocked by previous incubation with $25\,ng\,ml^{-1}$ of 8-OHdG, but not by over a thousand-fold greater concentration of guanosine. Further, enzymatic treatment with RNase did not affect the immunoreaction of oxidised DNA.

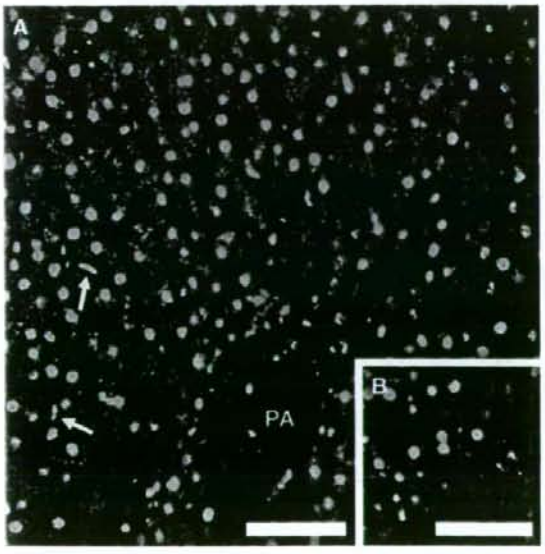

Figure 1 8-Hydroxydeoxyguanosine immunohistochemical staining in liver tissue from chronic hepatitis C and control (simple fatty liver) patients. (**A**) In the liver of chronic hepatitis C patient, 8-OHdG immunoreactivity was strongly observed throughout the whole acinus (PA = portal area) and mainly in the nuclei of hepatocytes and Kupffer cells (arrows in (**A**)). (**B**) In the liver of control (simple fatty liver), immunoreactivity of 8-OHdG was weak in the nuclei of hepatocytes. Scale bar, 100 μm in (**A**) and (**B**).

Analysis of factors associated with the occurrence of HCC in patients with chronic hepatitis C

Until the end of follow-up (mean was 6.7 ± 3.3 years), HCC occurrence was identified in 36 patients (30.5%) in this study. Seven patients died with no sign of HCC. The overall cumulative incidence of HCC was 3.4, 12.0, 17.2, and 38.9% at 1, 3, 5, and 10 years, respectively. To examine the effect of degree of liver oxidative DNA damage on HCC development during chronic HCV infection, clinical variables, including hepatic 8-OHdG quantification, were compared between patients who developed HCC and those who did not develop (non-HCC group) during the follow-up (Table 2). No significant difference was found in the patient age, body mass index, alcohol consumption, serum albumin levels, red blood cell count, and HCV genotype distribution between patients with and without HCC. In the group of patients with HCC, the proportion of male subjects, duration of infection, serum ALT, aspartate aminotransferase (AST), total bilirubin, hyaluronic acid, haemoglobin, iron, transferrin saturation, and ferritin levels at liver biopsy were significantly higher, and HCV-RNA titres and platelet count were significantly lower, than in the group of

Table 2 Comparison of epidemiologic and clinical variables of patients who developed HCC and patients who remained free of HCC during the follow-up period

Characteristics	HCC group ($N = 36$)	Non-HCC group ($N = 82$)	*P*-value
Age (years)	57.3 ± 8.2	54.7 ± 11.4	0.3718[a]
Gender (M/F)	26/10	40/42	**0.0182**[b]
Body mass index ($kg\,m^{-2}$)	23.6 ± 3.5	24.1 ± 3.2	0.6657[a]
Duration of HCV infection (years) ($N = 58$)	31.7 ± 10.5	26.9 ± 9.8	**0.0463**[a]
Alcohol intake ($g\,day^{-1}$)	21.0 ± 37.0	21.2 ± 38.9	0.6221[a]
Laboratory data			
ALT ($IU\,l^{-1}$)	91.9 ± 50.4	65.6 ± 52.9	**0.0021**[a]
AST ($IU\,l^{-1}$)	91.4 ± 42.7	60.5 ± 38.3	**0.0003**[a]
Serum albumin ($g\,dl^{-1}$)	3.65 ± 0.40	3.75 ± 0.45	0.1235[a]
Total bilirubin ($mg\,dl^{-1}$)	0.96 ± 0.29	0.75 ± 0.88	**< 0.0001**[a]
Hyaluronic acid ($ng\,ml^{-1}$)	206 ± 138	132 ± 151	**0.0003**[a]
Platelet count ($\times 10^4\,mm^{-3}$)	11.7 ± 4.5	16.4 ± 5.8	**< 0.0001**[a]
Red blood cell count ($\times 10^4\,mm^{-3}$)	429 ± 48	418 ± 50	0.1993[a]
Haemoglobin ($g\,dl^{-1}$)	13.9 ± 1.3	13.2 ± 1.6	**0.0302**[a]
Serum iron ($\mu g\,dl^{-1}$)	151 ± 68	121 ± 62	**0.0320**[a]
Transfemin saturation (%)	45.7 ± 22.6	36.2 ± 20.0	**0.0289**[a]
Serum ferritin ($ng\,ml^{-1}$)	264 ± 158	151 ± 149	**0.0002**[a]
Serum HCV-RNA ($kIU\,ml^{-1}$) ($N = 89$)	844 ± 900	1720 ± 1260	**0.0068**[a]
HCV genotype (1a/1b/2a/2b) ($N = 60$)	0/5/2/0	0/48/3/2	0.1100[b]
Liver histology			
Inflammatory activity (0/1/2/3)[c]	0/4/18/14	1/37/31/13	**0.0015**[b]
Fibrosis staging (0/1/2/3/4)[d]	0/1/3/10/22	1/28/23/17/13	**< 0.0001**[b]
Total iron score[e]	11.09 ± 4.75	6.23 ± 5.62	**< 0.0001**[a]
8-OHdG-positive hepatocytes (per $10^5\,\mu m^2$)	65.2 ± 20.2	40.0 ± 23.5	**< 0.0001**[a]

Data are expressed as mean ± s.d. HCC = hepatocellular carcinoma; ALT = alanine aminotransferase; AST = aspartate aminotransferase; HCV = hepatitis C virus; 8-OHdG = 8-hydroxydeoxyguanosine. [a]Mann–Whitney *U*-test. [b]Fisher's exact test, otherwise χ^2 test. [c]Inflammatory activity was graded according to the intensity of necroinflammatory lesions: 0, no histological activity; 1, mild activity; 2, moderate activity; 3, severe activity. [d]Fibrosis staging was scored as follows: 0, no fibrosis; 1, portal fibrosis without septa; 2, portal fibrosis with few septa; 3, numerous septa without cirrhosis; 4, cirrhosis. [e]The histological quantification of iron was assessed by total iron score proposed by Deugnier *et al* (1992).

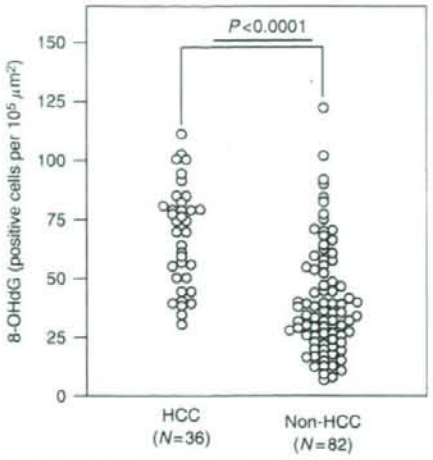

Figure 2 Comparison between 8-OHdG counts in patients who developed HCC ($N=36$) and those who remained free of HCC (non-HCC, $N=82$) during the follow-up period. Baseline 8-OHdG counts were significantly higher in the HCC group than in the non-HCC group in patients with chronic hepatitis C.

Table 3 Factors associated with the occurrence of HCC in patients with chronic hepatitis C by Cox proportional hazard regression analysis

Factor	Odds ratio	95% CI	P-value
Count of 8-OHdG-positive hepatocytes (each 10 cells per $10^5\,\mu m^2$ increase)	1.487	1.12–1.97	0.0058
Fibrosis staging (each stage 1 increase)	4.090	1.27–13.15	0.0181

HCC = hepatocellular carcinoma; 8-OHdG = 8-hydroxydeoxyguanosine; CI = confidence interval.

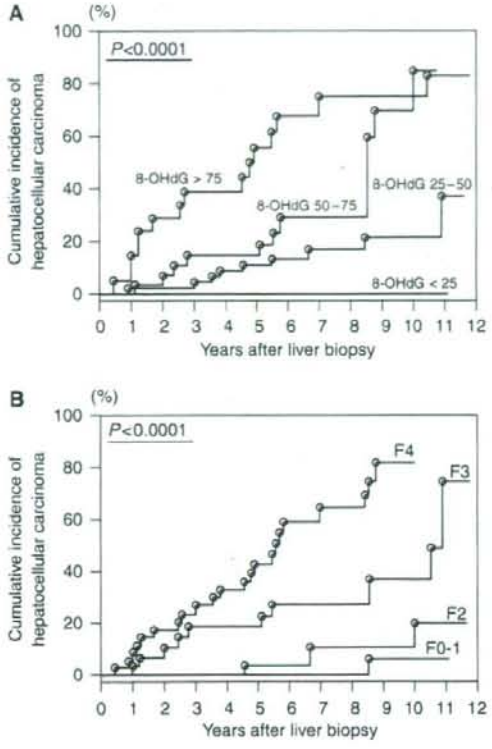

Figure 3 Cumulative incidence of HCC in 118 patients with chronic hepatitis C. Incidence curves were determined using the Kaplan–Meier method and statistical analysis was performed using the long-rank test. (**A**) Cumulative incidence of HCC divided by degrees of hepatic 8-OHdG expression levels. (**B**) Cumulative incidence of HCC divided by degrees of histological hepatic fibrosis staging score.

patients without HCC during the follow-up. The histological grading and staging scores were significantly higher in the HCC group than in the non-HCC group. The prevalence of hepatic iron deposits in patients with HCC was also significantly greater than that in non-HCC patients. Hepatic 8-OHdG expression levels in patients who developed HCC were significantly higher than in those who did not develop HCC (65.2 ± 20.2 *vs* 40.0 ± 23.5 positive cells per $10^5\,\mu m^2$, $P<0.0001$; Mann–Whitney *U*-test) (Figure 2). When hepatic steatosis was evaluated by scoring system as 0, no steatosis; 1, <33% of hepatocytes with steatosis; 2, 33–66% of hepatocytes affected; 3, >66% of hepatocytes affected, the degree of steatosis was not significantly different between HCC and non-HCC groups.

To examine the independent factors that affect the development of HCC, Cox proportional hazard regression analysis was performed using the 16 variables that were significantly different between HCC and non-HCC groups by univariate analyses. The multivariate analysis identified two factors as independent factors for HCC development: degree of hepatocytic 8-OHdG immunoreactivity (odds ratio, 1.487 (each 10 positive cells per $10^5\,\mu m^2$ increase); $P=0.0058$) and histological staging (odds ratio, 4.090 (each stage 1 increase); $P=0.0181$) (Table 3). When the patients were stratified according to the degree of hepatic 8-OHdG counts and histological fibrosis staging, the cumulative incidence of HCC was significantly increased in proportion to these variables (long-rank test) (Figure 3A and B). The cumulative incidences of HCC of 3, 5, and 10 years were 0, 0, and 0% in 8-OHdG counts of <25 (cells per $10^5\,\mu m^2$) subgroup ($n=23$), 4.4, 11.1, and 21.8% in 25–50 (cells per $10^5\,\mu m^2$) subgroup ($n=45$), 14.2, 14.2, and 84.9% in 50–75 (cells per $10^5\,\mu m^2$) subgroup ($n=29$), and 38.8, 55.5, and 74.6% in >75 (cells per $10^5\,\mu m^2$) subgroup ($n=21$), respectively.

Correlation between hepatocytic 8-OHdG counts and clinical characteristics in patients with chronic hepatitis C

To estimate the cause of hepatic oxidative DNA damage, correlation of clinical findings with hepatic 8-OHdG levels was evaluated (Table 4). The age of patients, body mass index, duration of infection, alcohol consumption, and the serum HCV-RNA titre were not related to the degree of oxidative DNA damage. 8-Hydroxydeoxyguanosine immunoreactivity was significantly higher in male than in female patients. Serum transaminases, platelet count, histological inflammation grade, and fibrosis stage were significantly correlated with the hepatic 8-OHdG levels. It is noteworthy that the hepatic 8-OHdG levels were strongly and positively correlated with body and hepatic iron deposition markers; serum ferritin levels and the hepatic iron deposit grade, that is, TIS, were strongly correlated with hepatic 8-OHdG count (8-OHdG *vs* ferritin, $r=0.640$, $P<0.0001$; *vs* TIS, $r=0.768$,

Table 4 Correlations between clinical findings and 8-OHdG levels in the liver of patients with chronic hepatitis C ($N=118$)

Characteristics	Hepatic 8-OHdG levels (positive cells per 10^5 μm^2)	Statistics r	Statistics P-values
Age (years)		0.149[a]	0.1059[a]
Gender			
Male ($N=66$)	57.7 ± 23.3		**<0.0001**[b]
Female ($N=52$)	36.7 ± 22.4		
Body mass index ($kg\,m^{-2}$)		0.073[a]	0.427[a]
Duration of HCV infection (years) ($N=58$)		0.237[a]	0.0677[a]
Alcohol intake ($g\,day^{-1}$)		0.121[a]	0.2709[a]
Laboratory data			
ALT ($IU\,l^{-1}$)		0.541[a]	**<0.0001**[a]
AST ($IU\,l^{-1}$)		0.605[a]	**<0.0001**[a]
Platelet count ($\times 10^4\,mm^{-3}$)		−0.430[a]	**<0.0001**[a]
Serum ferritin ($ng\,ml^{-1}$)		**0.640**[a]	**<0.0001**[a]
Serum HCV-RNA ($kIU\,ml^{-1}$) ($N=89$)		−0.197[a]	0.0721[a]
Inflammatory activity[c]			
A0 or A1 ($N=42$)	32.2 ± 21.2		
A2 ($N=49$)	52.3 ± 22.6		**<0.0001**[d]
A3 ($N=27$)	62.4 ± 26.0		
Fibrosis staging[e]			
F0 or F1 ($N=30$)	26.6 ± 14.7		
F2 ($N=26$)	46.0 ± 22.1		**<0.0001**[d]
F3 ($N=27$)	52.4 ± 21.2		
F4 ($N=35$)	66.3 ± 26.3		
Total iron score[f]		**0.768**[a]	**<0.0001**[a]

Data are expressed as mean ± s.d. 8-OHdG = 8-hydroxydeoxyguanosine; HCV = hepatitis C virus; ALT = alanine aminotransferase; AST = aspartate aminotransferase. [a]Spearman rank correlation test. [b]Mann–Whitney *U*-test. [c]Inflammatory activity was graded according to the intensity of necroinflammatory lesions: 0, no histological activity; 1, mild activity; 2, moderate activity; 3, severe activity. [d]Kruskal–Wallis test. [e]Fibrosis staging was scored as follows: 0, no fibrosis; 1, portal fibrosis without septa; 2, portal fibrosis with few septa; 3, numerous septa without cirrhosis; 4, cirrhosis. [f]The histological quantification of iron was assessed by total iron score proposed by Deugnier *et al* (1992).

$P<0.0001$) (Table 4 and Figure 4). These results suggest the association between hepatic 8-OHdG production and iron deposition in the liver of patients with chronic hepatitis C.

DISCUSSION

Although free radicals are normally produced by many reactions essential for cell metabolism and energy production, they are also implicated in the pathogenesis of several different diseases (Valko *et al*, 2007). Reactive oxygen species production within the cells is controlled by numerous antioxidant intracellular defence mechanisms, but under certain conditions, ROS overproduction exceeds the cellular defences and damages cell components including nucleic acids (Valko *et al*, 2007). Reactive oxygen species attack on DNA causes the production of stable covalent bonds and the subsequent formation of DNA adducts, such as 8-OHdG (Shigenaga *et al*, 1989). Experiments in which DNA templates containing 8-OHdG were used indicated that this oxidatively modified DNA residue can induce G-C to T-A transversion at DNA replication (Kuchino *et al*, 1987; Shibutani *et al*, 1991), suggesting that the lesion is mutagenic and therefore potentially carcinogenic, but the role of this oxidative DNA adduct in human carcinogenesis is not entirely understood.

Chronic HCV infection is recognised as the most major risk factor for HCC (Nishioka *et al*, 1991; El-Serag, 2002; Seeff, 2002), but little is known about the precise role of HCC development in HCV-related liver disease. It was reported recently that oxidative damage is a peculiar feature of HCV-mediated liver injury. Patients with chronic hepatitis C showed increased oxidative stress markers in serum or in the liver (Shimoda *et al*, 1994; Sumida *et al*, 2000; Mahmood *et al*, 2004; Fujita *et al*, 2007). Therefore, we measured the amount of 8-OHdG in liver biopsy specimen of patients with chronic hepatitis C and examined its relation with future HCC development. Baseline clinical variables were compared between patients with and without HCC development. Based on univariate analysis, the following numerous variables were picked up for potential factors for HCC development: (1) gender and duration of infection, (2) hepatic inflammation (serum ALT and AST levels and histological grade), (3) hepatic fibrosis (hyaluronic acid, platelet count, and histological stage), (4) iron-related markers (haemoglobin, serum iron, transferrin saturation, ferritin, and TIS), (5) serum HCV-RNA titres, and (6) hepatocytic 8-OHdG

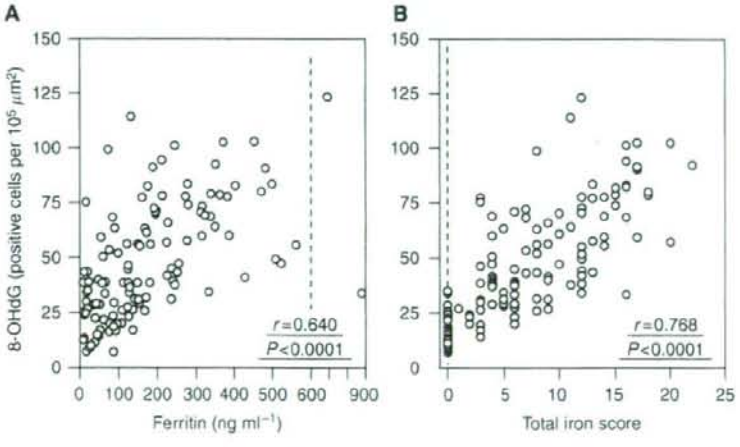

Figure 4 Correlations between hepatic 8-OHdG staining and serum ferritin levels (**A**), and TIS in hepatic tissues (**B**), in 118 patients with chronic hepatitis C.

counts. Cox proportional hazard analysis identified increased hepatic oxidative DNA damage, together with histological fibrosis, which is a well-recognised risk factor for HCC (Greten *et al*, 2005), as an independent risk factor for HCC development. This result suggests that the hepatic oxidative stress plays an important role in hepatocarcinogenesis and it may be a useful marker to predict future HCC development in chronic HCV-infected patients. Especially in the group of patients with hepatic 8-OHdG counts exceeding 75 positive cells per $10^5\ \mu m^2$, the HCC incidence during the first 3 years was extremely high (38.8%) (Figure 3A), indicating that these patients constitute a very high-risk subgroup for developing HCC and should necessarily be carefully monitored by several modalities. Recently, Maki *et al* (2007) evaluated the expression levels of 8-OHdG of non-cancerous hepatic tissues in HCV-infected patients who developed HCC and received curative tumour resection. The postoperative cumulative HCC-free survival was significantly shorter in patients with the highest percentage of 8-OHdG-positive hepatocytes, indicating that the hepatic 8-OHdG levels are also useful for prediction of HCC recurrence in patients with chronic HCV infection who developed HCC.

Several additional risk factors for HCC were identified in patients with chronic hepatitis C in previous reports – increased age (Seeff, 2002), heavy alcohol intake (Donato *et al*, 1997), and chronic coinfection with HBV (Donato *et al*, 1997) – but our results did not identify these factors for HCC. This may be attributable to the fact that our study population excluded heavy alcohol abusers (defined as a chronic consumer of ethanol in excess of 40 g day^{-1} for male and 20 g day^{-1} for female for at least 5 years) and included relatively old patients (median age was 57.5 years). Patients coinfected with HBV were completely excluded from our study because all patients were seronegative for both hepatitis B surface antigen and hepatitis B core antibody. Recently, several reports have suggested that persons with diabetes mellitus are at an increased risk for developing HCC (El-Serag *et al*, 2001), and obesity, which frequently accompanies diabetes, has also been reported to increase the risk for hepatic steatosis and HCC in HCV-infected patients (Ohata *et al*, 2003). But, body mass index and hepatic steatosis were not significantly different between the HCC- and non-HCC-developed groups among our patients.

To determine the factors involved in the occurrence of hepatic oxidative stress during chronic HCV infection, epidemiologic, laboratory, and histological variables were examined for association with hepatocytic 8-OHdG staining counts. Quantitative analysis revealed that hepatocytic 8-OHdG levels were significantly correlated with serum aminotransferase levels and with the histological grading of necroinflammation, suggesting a possible link between hepatic oxidative stress and hepatic inflammation in chronic hepatitis C. It is unclear whether oxidative stress is the cause or the consequence of liver injury, but it has been demonstrated that oxidative stress can directly activate Kupffer cells, causing the release of inflammatory and profibrogenic cytokines such as tumour necrosis factor-α and transforming growth factor-β (Poli and Parola, 1997). Accordingly, the hepatocytic 8-OHdG counts were also significantly correlated with hepatic fibrosis, as assessed by the serum hyaluronic acid, platelet count, and histological staging score. Sumida *et al* (2000) have also shown a significant association between oxidative stress (serum thioredoxin levels) and hepatic fibrosis (hyaluronic acid, type IV collagen-7S domain, procollagen-III peptide) in HCV-positive persons, suggesting that ROS is an important cofactor in accelerating the development of hepatic fibrosis during chronic HCV infection, which may lead to further acceleration of HCC development. In addition, the hepatic 8-OHdG levels were significantly correlated with the serum ferritin and hepatic iron amounts assessed by TIS, suggesting a strong relationship between the damage to hepatocytic DNA and body store of iron in chronic hepatitis C patients. It is known that free iron promotes generation of oxygen radicals by catalysing the Fenton reaction in which Fe^{2+} reacts with H_2O_2 to generate highly reactive $OH^{\bullet}$ radicals, which can cause nucleic acid damage and 8-OHdG adducts. Therefore, iron may cause liver tissue injury by increasing the formation of toxic hydroxyl radicals leading to progression of liver inflammation, fibrosis, and increased risk for developing liver cancer during chronic HCV infection. Increased iron stores were associated with increased oxidative DNA damage, suggesting that removing iron stores in the body, for example by phlebotomy (Yano *et al*, 2004), or dietary iron restriction (Iwasa *et al*, 2004), which has been accepted as a useful treatment option, may delay or reduce the incidence of HCC in patients with chronic hepatitis C. Additional studies are warranted to determine whether these iron-reduction therapies are effective for reducing HCC and for improving the clinical outcomes of patients with chronic HCV infection.

In conclusion, this study clearly showed that in patients with chronic hepatitis C, the oxidative DNA damage in the liver frequently occurred and that it was strongly associated with increased risk for HCC. Strong positive correlations between hepatic oxidative stress and iron overload suggest that iron content may be a mediator of hepatic oxidative stress and that iron reduction may be beneficial to reduce the HCC incidence in chronic HCV-infected patients.

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Comparison of hepatic oxidative DNA damage in patients with chronic hepatitis B and C

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SUMMARY. 8-Hydroxydeoxyguanosine (8-OHdG) is a promutagenic DNA lesion produced by hydroxyl radicals and is recognized as a useful marker in estimating DNA damage induced by oxidative stress. The aim of this study was to clarify the clinical significance of hepatic 8-OHdG levels in patients with chronic viral hepatitis. Hepatic 8-OHdG accumulation was investigated in patients with chronic hepatitis C (CH-C) ($n = 77$) and chronic hepatitis B (CH-B) ($n = 34$) by immunohistochemical staining of liver biopsy samples. 8-OHdG positive hepatocytes were significantly higher in patients with CH-C compared to CH-B (median 55.0 *vs* 18.8 cells/10^5 μm^2, $P < 0.0001$). The number of positive hepatocytes significantly increased with the elevation of serum aminotransferase levels, especially in CH-C patients (8-OHdG *vs* alanine aminotransferase (ALT)/aspartate aminotrasferase (AST) were $r = 0.738/0.720$ in CH-C and 0.506/0.515 in CH-B). 8-OHdG reactivity was strongly correlated with body and hepatic iron storage markers in CH-C (*vs* serum ferritin, $r = 0.615$; *vs* hepatic total iron score, $r = 0.520$; *vs* hepatic hepcidin mRNA levels, $r = 0.571$), although it was related to serum HBV-DNA titers ($r = 0.540$) and age of patients ($r = -0.559$) in CH-B. These results indicate that hepatic oxidative DNA damage is common in chronic viral hepatitis, in particular chronic HCV-infected patients, suggesting a possible link between chronic hepatic inflammation and hepatocarcinogenesis. The strong positive correlation between hepatic DNA damage and iron overload suggests that iron content is one of the most likely mediators of hepatic oxidative stress and iron reduction may be beneficial to reduce the incidence of hepatic cancer in CH-C patients.

Keywords: alanine aminotransferase/aspartate aminotrasferase; hepatitis B virus, hepatitis C virus, hepatocellular carcinoma, iron, oxidative stress.

INTRODUCTION

Reactive oxygen species (ROS) in living cells have been implicated in a number of pathologies, including aging, inflammatory diseases and the development of cancer, because they cause oxidative damage to nucleic acids, proteins, and lipids [1]. ROS include oxygen-centred radicals and non-radical compounds. Among the many radicals, the hydroxyl radical is the most reactive and is also responsible for the formation of 8-hydroxydeoxyguanosine (8-OHdG) [2,3]. 8-OHdG is known to induce G-C to T-A transversions during DNA replication [4]. Therefore, 8-OHdG is considered a useful marker for oxidatively generated DNA damage, leading to carcinogenesis [3].

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide, and the death rate due to this tumour has been increasing over the past

Abbreviations: 8-OHdG, 8-hydroxydeoxyguanosine; CH-B, chronic hepatitis B; CH-C, chronic hepatitis C; EIA, enzyme immunoassay; ELISA, enzyme-linked immunosorbent assay; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HIS, hepatic iron score; mRNA, messenger RNA; NO, nitric oxide; NOS2, nitric oxide synthase 2; NSAIDs, nonsteroidal anti-inflammatory drugs; PBS, phosphate buffered saline; PCR, polymerase chain reaction; PIS, portal iron score; ROS, reactive oxygen species; SIS, sinusoidal iron score; TfR2, transferrin receptor 2; TIS, total iron score; TMA, transcription-mediated amplification.

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20–30 years in the Unites States [5] and in Japan [6]. It has been shown that chronic hepatitis C virus (HCV) and hepatitis B virus (HBV) infections are strong and independent risk factors associated with the likelihood of HCC [7,8], but the precise mechanism(s) of hepatocarcinogenesis during chronic viral hepatitis in humans are still largely unknown. Recently, there has been an increasing body of evidence suggesting that oxidative stress may play a pathogenic role in chronic hepatitis, especially in patients with chronic hepatitis C (CH-C). It has been demonstrated that plasma samples from HCV-infected patients have increased lipid peroxidation products [9], superoxide dismutase activity [10], and 8-OHdG level in circulating leukocyte DNA [11]. Immunohistochemistry has also documented the presence of oxidative stress formation in the livers of CH-C patients [9,12]. Involvement of oxidative stress in pathogenesis during chronic HCV infection is also supported by the fact that antioxidant therapy improved liver injury caused by HCV infection [11,13]. Despite these evidences, little is understood about the mechanisms of oxidative stress formation during chronic HCV infection.

In the case of chronic HBV infection, mechanisms of hepatocarcinogensis have been proposed by using several theoretical roles. Early studies suggested that the direct integration of the HBV genome into human chromosomes might cause inactivation of tumour suppressor/proto-oncogenes [14]. The HBV encoded X protein has been shown to initiate transactivation as well as induction of signal transduction pathways such as Ras/Raf-1 [15], and the large surface HBV protein has been shown to induce HCC in a transgenic mouse model [16]. But the pathogenic role(s) and clinical significance of oxidative stress formation in the livers of patients with chronic hepatitis B (CH-B) has not been sufficiently investigated.

To examine the potential role of hepatic oxidative stress formation for the pathogenesis of liver injury caused by chronic HCV and HBV infections, we compared 8-OHdG-positive hepatocyte count in liver biopsy specimens of CH-C and CH-B patients, and evaluated the association between hepatic oxidative DNA damage and demographic, biochemical and histological findings.

PATIENTS AND METHODS

Patients with CH-C and CH-B

A total of 111 consecutive non-selected patients with CH-C and CH-B who underwent needle liver biopsy at Mie University Hospital between March 1998 and September 2005 were enrolled in this study. About 77 of them had CH-C and included 45 males and 32 females, with a median age of 55.0 (range, 25–81) years. Diagnosis of chronic HCV infection was based on the consistent detection of serum anti-HCV antibody [the third-generation enzyme-linked immunosorbent assay (ELISA): Ortho Diagnostic Systems, Raritan, NJ, USA] and HCV-RNA (Amplicor-HCV assay: Roche Molecular Diag. Co., Tokyo, Japan). Serum HCV-RNA titre was quantified by the Amplicor monitor assay (Roche Molecular Diag. Co.). About 34 patients with CH-B [24 males and 10 females, with a median age of 54.0 (range, 24–75) years] who underwent liver biopsy during the same period were also recruited. All CH-B patients were positive for both HBsAg and anti-HBc [commercial enzyme immunoassay kits (EIA): Abbott Laboratories, North Chicago, IL, USA], and 22 patients (64.7%) were HBeAg (EIA: Abbott Laboratories) positive. Serum HBV-DNA titre was measured using the transcription-mediated amplification (TMA) assay (Mitsubishi Kagaku BML, Tokyo, Japan). Patients with other liver diseases (drug-induced, autoimmune and metabolic) were excluded by appropriate serological testing and clinical history. None of the patients were co-infected with both HBV and HCV, or received any antiviral or immunomodulatory treatment in the preceding 6 months of the study. Patients with concurrent diseases or those taking medications capable of interfering with free radical production, such as nonsteroidal anti-inflammatory drugs (NSAIDs), vitamins and iron-containing drugs were also excluded.

The following parameters were obtained form each patient at the time of liver biopsy: age; sex; body mass index; alcohol intake; biochemical, haematological, iron-related [serum iron, transferrin saturation (calculated and expressed as a percentage: serum iron/total iron binding capacity × 100%), and ferritin levels], and virological serum markers; liver histological findings; presence of hepatic 8-OHdG; and hepatic messenger RNA (mRNA) levels of transferrin receptor 2 (TfR2) and hepcidin. Informed consent was obtained from each patient included in the study and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a *priori* approval by the Ethical Committee of Mie University.

Histological evaluation

Liver biopsy specimens were immediately divided in to two parts, and one portion was fixed in 10% buffered formalin and embedded in paraffin for routine histological examination and the other was frozen and stored at −80 °C for RNA extraction. The former samples were stained with haematoxylin–eosin and Masson's trichrome, and were graded for the degree of necroinflammatory activity and staged for the extent of fibrosis using the criteria of Desmet *et al.* [17]. The histological quantification of hepatic iron using liver samples stained with Perls' Prussian blue was carried out according to Deugnier *et al.* [18] by scoring iron separately within hepatocytes [Hepatic Iron Score (HIS), 0–36], sinusoidal cells [Sinusoidal Iron Score (SIS), 0–12], and portal tracts [Portal Iron Score (PIS), 0–12]. The Total Iron Score (TIS, 0–60) represented the sum of these scores. This score has been

shown to correlate highly with the biochemical hepatic iron index and hepatic iron concentration measured by atomic absorption spectrophotometry in patients with chronic liver disease [19–21].

Immunohistochemical detection of 8-OHdG adducts in liver biopsy samples

Immunohistochemical staining of 8-OHdG was performed as previously described [22]. A mouse monoclonal antibody against 8-OHdG (Japanese Aging Control Institute, Shizuoka, Japan) and Alexa 488-labelled goat anti-mouse IgG (Molecular Probes, Eugene, OR, USA) were used. The degree of immunoreactivity was estimated by counting the number of stained hepatocyte nuclei using Adobe Photoshop version 5.5 and NIH Image free software (Vers. 1.62, National Institute of Health, image program) [22]. The specificity of this anti-8-OHdG monoclonal antibody was confirmed by (i) comparison with adjacent sections in which the primary antibody was omitted, or (ii) using normal mouse serum instead of the primary antibody, or (iii) absorption with purified 8-OHdG (Sigma, Tokyo, Japan) or guanosine (Sigma), or (iv) RNA digestion. The primary antibody was incubated for 5 h at room temperature with serial dilutions of purified 8-OHdG or guanosine in phosphate buffered saline (PBS) ranging from 2.5 mg/mL through 2.5 ng/mL and applied to the sections. RNA digestion was performed before the immunostaining procedures in PBS containing DNase-free RNase (5 μg/μL) for 1 h at 37 °C.

TfR2 and hepcidin mRNA quantification in liver biopsy samples

Hepatic mRNAs of TfR2 and hepcidin were measured using the TaqMan real-time detection-polymerase chain reaction (PCR) assay (Applied Biosystems, Tokyo, Japan), as previously described [23,24]. mRNA was extracted from liver biopsy specimens using the SV-RNA Isolation System (Promega corporation, Madison, USA). Primer and probe sequences are shown in Table 1. The results for TfR2 and hepcidin mRNA were expressed as the amount relative to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA quantified simultaneously in each liver sample.

Statistical analysis

Results were expressed as median with range. Comparisons between groups were performed using the Mann–Whitney *U*-test or the Kruskal–Wallis test for continuous variables and the chi-square or Fisher's exact test for categorical data. Correlation coefficients between numerical variables were calculated as Spearman's rank test. All tests were two-tailed and P values < 0.05 were considered as statistically significant. Statistical analysis was performed using the commercially available software SPSS 11.5 software (SPSS Inc., Chicago, IL, USA).

Table 1 List of primers and probes used for real-time detection-PCR Assay

Primers and probes	Sequence
TfR2	
Forward primer	5' -TGGTGACTTTGGAAGCGTG-3'
Reverse primer	5'-CTGGTCTTGGCATGAAACTTG-3'
Probe	5-FAM-TAGTGTACGTGAGCCTGGA-CAACGCAGT-TAMRA-3'
Hepcidin	
Forward primer	5'-TTCCCCATCTGCATTTTCTG-3'
Reverse primer	5'-TCTACGTCTTGCAGCACATCC-3'
Probe	5'-FAM-TGCGGCTGCTGTCATCGAT-CAA-TAMRA-3'
GAPDH	
Forward primer	5'-GAAGGTGAAGGTCGGAGTC-3'
Reverse primer	5'-GAAGATGGTGATGGGATTC-3'
Probe	5'-FAM-CAAGCTTCCCGTTCTCAG-CC-TAMRA-3'

PCR, polymerase chain reaction; TfR2, transferrin receptor 2; FAM, 6-carboxyfluorecein; TAMRA, 6-carboxytetramethyl-rhodamine; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

RESULTS

Comparison of clinical profiles of patients with CH-C and CH-B

Demographic and laboratory data and histological features of patients with CH-C and CH-B are shown in Table 2. There was no significant difference in the median age and gender distribution between the HCV and HBV groups. Laboratory data, except for serum ferritin, were not significantly different between the two groups. CH-C patients had significantly higher ferritin levels compared to CH-B [211.6 (range 13.2–792) *vs* 101.9 (9.1–322) ng/mL, $P = 0.0032$]. Among the 77 CH-C patients, serum ferritin levels were elevated above the normal range (> 220 ng/mL for male and > 100 ng/mL for female) in 45 cases (58.4%) in contrast to 11 cases (32.4%) from 34 CH-B patients, and this reached statistical significance ($P = 0.0198$). Liver histology showed no significant difference in grading and staging score between the CH-C and CH-B patients. Iron deposition in the liver was more prominent in CH-C patients; TIS was significantly higher in CH-C compared to CH-B patients [7.0 (0–22) *vs* 3.0 (0–16), $P = 0.0033$]. Hepatic TfR2 mRNA levels were significantly higher in CH-C patients than in CH-B [3740 (237–30 700) *vs* 2095 (66.7–72 100), $P = 0.0055$].

Table 2 Clinical characteristics of patients with chronic hepatitis C and B

Characteristics	Chronic hepatitis C (n = 77)	Chronic hepatitis B (n = 34)	P-Value
Age (years)	55 (25–81)	54 (24–75)	NS
Gender (M/F)	45/32	24/10	NS
Body mass index (kg/m^2)	24.0 (16.5–31.3)	24.0 (18.3–28.2)	NS
Laboratory data			
ALT (IU/L)	56 (19–411)	49 (11–561)	NS
AST (IU/L)	60 (19–565)	58.5 (18–702)	NS
Hyaluronic acid (ng/mL)	58.7 (9.0–649)	49.0 (11.0–324)	NS
Platelet count (× 10^4/mm^3)	14.9 (4.9–29.8)	14.3 (3.6–25.3)	NS
Red blood cell count (× 10^4/mm^3)	425 (234–565)	447.5 (280–566)	NS
Haemoglobin (g/dL)	13.7 (7.9–16.8)	13.8 (9.2–17.1)	NS
Serum iron (μg/dL)	128 (32–228)	128.5 (20–240)	NS
Transferrin saturation (%)	39.1 (8.5–85.4)	41.5 (6.1–85.4)	NS
Serum ferritin (ng/mL)	211.6 (13.2–792)	101.9 (9.1–322)	0.0032
Viral titre			
HCV-RNA (KIU/mL)	682 (10.4–5100)	–	
HBV-DNA (LGE/mL)	–	5.4 (<3.7–8.7)	
Liver histology			
Inflammatory activity (0/1/2/3)*	1/28/42/6	1/12/14/7	NS
Fibrosis staging (0/1/2/3/4)†	2/25/21/15/14	0/6/10/9/9	NS
Total iron score‡	7 (0–22)	3 (0–16)	0.0033
TfR2 mRNA (/GAPDH)	3740 (237–30 700)	2095 (66.7–72 100)	0.0055
Hepcidin mRNA (/GAPDH)	4500 (230–35 200)	3290 (250–41 000)	NS

Data are expressed as median (range).
*Inflammatory activity was graded according to the intensity of necroinflammatory lesions: 0, no histological activity; 1, mild activity; 2, moderate activity; 3, severe activity.
†Fibrosis staging was scored: 0, no fibrosis; 1, portal fibrosis without septa; 2, portal fibrosis with few septa; 3, numerous septa without cirrhosis; 4, cirrhosis.
‡Histological quantification of iron was assessed by total iron score proposed by Deugnier *et al* (1992).
ALT, alanine aminotransferase; AST, aspartate aminotransferase; TfR2, transferrin receptor 2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

although the hepcidin levels were not significantly different between the two groups, as reported previously [23,24].

In situ detection of 8-OHdG positive hepatocytes using liver biopsy samples

Figures 1a,b show the 8-OHdG immunohistochemical staining in liver biopsy samples in patients with CH-C and CH-B. 8-OHdG immunoreactivity was strongly observed in the nuclei (and weekly in the cytoplasm) of hepatocytes. Kupffer cells and infiltrated inflammatory cells in CH-C liver biopsies (Fig. 1a). The hepatocyte nuclei were differentiated from the nuclei of other cells using computed analyses based on nuclear shape and size. 8-OHdG-immunoreactive hepatocytes were distributed throughout the whole acinus in the livers of patients. Using the liver samples of patients with CH-B, relatively faint immunoreactivity of 8-OHdG was observed in the nuclei of hepatocytes and was rarely in the cytoplasm (Fig. 1b). As a whole, 8-OHdG-positive hepatocyte counts were significantly higher in CH-C patients than in CH-B [55.0 (range 12–126) *vs* 18.8 (6.3–138) cells/10^5 μm^2, $P < 0.0001$] (Fig. 1c). Stratifying the patients according to the histological inflammatory grade, in the mild and moderate hepatitis subgroup, CH-C patients had significantly higher hepatic 8-OHdG levels than CH-B [in grade 0/1, 47.3 (12–126) *vs* 16.3 (6.3–36.7) cells/10^5 μm^2, $P < 0.0001$; in grade 2, 54.7 (21.3–121) *vs* 18.2 (6.7–39.3) cells/10^5 μm^2, $P < 0.0001$] (Fig. 1d).

The specificity of the anti-8-OHdG antibody used in this study was confirmed by several parallel experiments. Sections in which the primary antibody was omitted or those treated with normal control serum instead of the primary 8-OHdG antibody consistently yielded negative staining. Localization of 8-OHdG was considered specific because the recognition of hepatocytes was completely blocked by previous incubation with 25 ng/mL of 8-OHdG, but not by

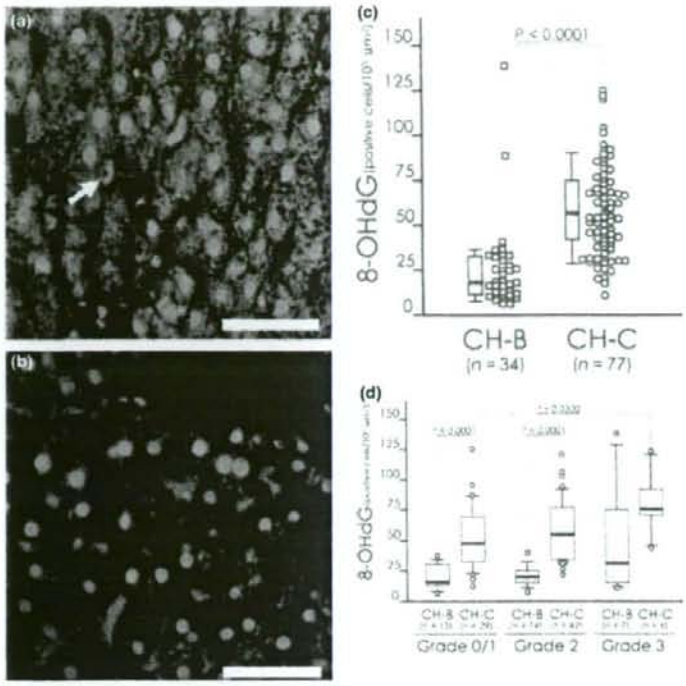

Fig. 1 Representative 8-hydroxydeoxyguanosine (8-OHdG) immunohistochemical staining in liver tissues from patients with chronic hepatitis C (CH-C) (a) and chronic hepatitis B (CH-B) (b). In the liver of CH-C patient, 8-OHdG immunoreactivity was strongly observed throughout the whole acinus and mainly in the nuclei of hepatocytes and Kupffer cells [arrow in (a)]. In the liver of CH-B patient, the relatively faint immunoreactivity of 8-OHdG was observed in the nuclei of hepatocytes and rarely in the cytoplasm. Scale bar 100 μm. (c) Comparison between 8-OHdG-positive hepatocytic nuclear count in patients with CH-C and CH-B. Positive cells were significantly higher in CH-C patients than in CH-B. Box and whisker graphs depict the median (line within the box), 25–75 percentiles (upper and lower border of the box), and 10–90 percentiles (whiskers). Open circles and squares refer to patients with CH-C and CH-B respectively. (d) 8-OHdG-positive hepatocytic counts were stratified according to histological grading score in patients with CH-C and CH-B. Box and whisker graphs depict the median (line within the box), 25–75 percentiles (upper and lower border of the box), and 10–90 percentiles (whiskers), with outliners plotted individually.

over a thousand-fold greater concentration of guanosine. Further, enzymatic treatment with RNase did not affect the immunoreaction of oxidized DNA.

Clinical variables that correlate with hepatic oxidative DNA damage in patients with CH-C

To estimate the source of oxidative-generated DNA damage frequently occurring in the livers of patients with CH-C, the correlation of clinical and histological findings with the degree of hepatic damaged DNA was evaluated, and the results are summarized in Table 3. The age of patients was not related to the degree of hepatic oxidative DNA damage in CH-C patients. Serum transaminase levels were significantly correlated with hepatic 8-OHdG levels in patients with CH-C (8-OHdG *vs* ALT, $r = 0.738$, $P < 0.0001$; *vs* AST, $r = 0.720$, $P < 0.0001$) (Table 3; Figs 2a,b). Hepatic 8-OHdG was significantly higher in a subgroup of CH-C patients with histologically advanced to severe hepatitis (grade 3) than in those with mild hepatitis (grade 0/1) [74.5 (43–123) *vs* 47.3 (12–126) cells/10^5 μm^2, $P = 0.0320$] (Fig. 1d). It is noteworthy that the hepatic 8-OHdG levels were strongly and positively correlated with body and hepatic iron deposition markers in patients with CH-C; serum ferritin levels and the hepatic iron deposit grade, i.e. TIS, were strongly correlated with 8-OHdG-positive hepatocyte nuclei (8-OHdG *vs* ferritin, $r = 0.615$, $P < 0.0001$; *vs* TIS, $r = 0.520$, $P < 0.0001$) (Table 3; Figs 2c,d). Hepcidin, that is exclusively synthesized in the liver, was recently identified as a key regulatory hormone of iron homeostasis, and is reported to be up-regulated in response to iron overload [24–26]. Therefore, we evaluated the relation of 8-OHdG levels with