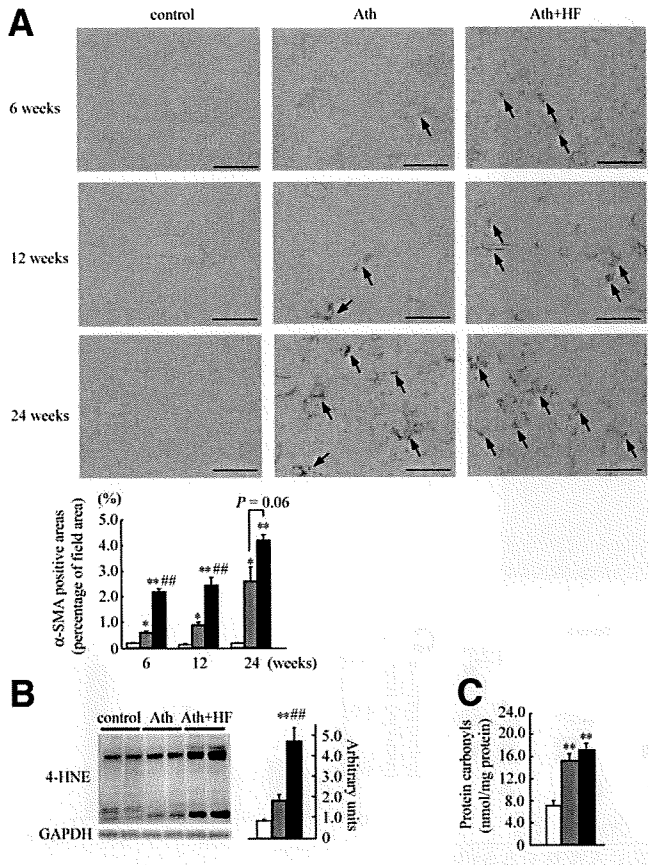

Fig. 5. Activation of HSCs and oxidative stress in the livers of mice fed the Ath or Ath+HF diet. (A) Hepatic α-SMA-positive cells (indicated by arrows) were detected by immunohistochemical staining at 6, 12, or 24 weeks. The original magnification was ×200. The scale bars represent 10 μm. The α-SMA-positive area was quantified morphometrically in the liver sections of mice fed standard chow (white bar; n = 3), the Ath diet (gray bar; n = 3), or the Ath+HF diet (black bar; n = 3) at different times, as described in the Materials and Methods section. (B) Western blot of 4-HNE-modified proteins in the liver after 24 weeks. The hepatic content of 4-HNE-modified proteins was quantified in mice fed standard chow (white bar; n = 4), the Ath diet (gray bar; n = 4), or the Ath+HF diet (black bar; n = 4), as described in the Materials and Methods section. (C) Hepatic protein carbonyls were determined in the mice fed standard chow (white bar; n = 3), the Ath diet (gray bar; n = 4), or the Ath+HF diet (black bar; n = 4) after 24 weeks, as described in the Materials and Methods section. The values represent the means ± the SEM. *$P < 0.05$ and **$P < 0.01$ versus the control group. #$P < 0.05$ and ##$P < 0.01$ versus the Ath group.

element binding protein 1c (SREBP-1c), a transcriptional regulator of fatty acid synthesis,[25] and fatty acid synthase (FAS), was coordinately up-regulated. In contrast, the expression levels of genes for the mitochondrial fatty acid β-oxidation pathway were coordinately repressed in concert with a decrease in the expression of peroxisome proliferator-activated receptor α (PPARα), a transcriptional up-regulator of fatty acid β-oxidation in the liver.[26] It is recognized that mitochondrial β-oxidation and the levels of carnitine palmitoyltransferase 1a (CPT-1a) and PPARα expression are increased compensatively in the livers of patients with NAFLD[27,28] and obese-diabetic (ob/ob) mice with severe steatosis of the liver.[29] Therefore, although the levels of PPARα and CPT-1a mRNA expression in the Ath+HF group were higher than those in the Ath group, it may not have been enough to metabolize the excessive fatty acids from the high-fat component and intrahepatic fatty acid synthesis.

It is believed that oxidative stress due to the generation of reactive oxygen species (ROS) or decreased antioxidant defenses is directly involved in the development of steatohepatitis.[30] The expression levels of genes for the reduced-form nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex, an important source of ROS,[31] were coordinately elevated in mice fed the Ath diet and further up-regulated in mice fed the Ath+HF diet.

The Ath diet has previously been reported to induce the expression of genes for inflammation.[32,33] Our results further demonstrate that inflammatory cytokines, such as tumor necrosis factor α (TNF-α), chemokines, and their receptors, are up-regulated in mice fed the Ath diet.

The Ath diet also induced genes involved in collagen accumulation, especially after 24 weeks. At 6 weeks, the expression levels of collagen genes were higher in the Ath+HF group than in the Ath group (Fig. 6). In addition, the expression levels of genes for TGF-β and plasminogen activator inhibitor 1 (PAI-1), key inducers of fibrogenesis, were dramatically up-regulated in the Ath+HF group compared with the Ath group at 24 weeks. These results support the finding that the Ath+HF diet induces more rapid progression of steatohepatitis than the Ath diet.

Discussion

Whether cholesterol, TG, or FFA contributes to the development of NASH remains controversial.[34] Because the feeding of cholesterol and cholic acid, which are the main components of the Ath diet, leads to the additive accumulation of cholesterol in the liver, the main pathology in Ath diet–induced steatohepatitis is caused by cholesterol-induced toxicity.[35]

In this study, we have shown that Ath diet–induced steatohepatitis with atherosclerosis is a better experimental model of human NASH for the following reasons: (1) this model seems to be a more physiological dietary model of NASH than existing animal models, which require genetic defects, chemical agents such as carbon tetrachloride, or the depletion of nutrients, such as the MCD diet–induced model; (2) the liver pathology involves steatohepatitis with cellular ballooning, a necessary histological feature defining human NASH; (3) the addition of a high-fat component to the Ath diet causes hepatic insulin

Table 3. Biological Pathways of Liver Genes Regulated by the Ath or Ath+HF Diets After 6 or 24 Weeks

Pathway Name	Number of Genes Changed	Number of Genes Measured	Z Score	Permuted *P* Value
Ath diet				
Up-regulated at 6 weeks				
Inflammatory Response	23	41	3.22	< 0.01
DNA replication Reactome	21	41	2.56	0.010
Cell Cycle-G1 to S control Reactome	32	68	2.56	0.016
G1 to S cell cycle Reactome	32	68	2.56	0.016
RNA transcription Reactome	20	40	2.36	0.036
p38 MAPK signaling	15	28	2.38	0.037
Down-regulated at 6 weeks				
Amino Acid Metabolism	23	45	2.94	< 0.01
Cholesterol Biosynthesis	11	15	3.56	< 0.01
Complement and Coagulation Cascades	29	59	3.04	< 0.01
Mitochondrial fatty acid betaoxidation	11	16	3.28	< 0.01
Blood Clotting Cascade	11	18	2.77	0.012
Unsaturated Fatty Acid Beta Oxidation	5	6	2.78	0.014
Biogenic Amine Synthesis	8	14	2.13	0.042
Krebs-TCA Cycle	14	29	2.03	0.045
Up-regulated at 24 weeks				
mRNA processing binding Reactome	196	438	5.91	< 0.01
TGF Beta Signaling Pathway	62	124	4.37	< 0.01
Translation Factors	27	49	3.50	< 0.01
Complement Activation Classical	9	15	2.34	0.021
Down-regulated at 24 weeks				
GPCRDB Other	52	147	3.58	< 0.01
Small ligand GPCRs	11	19	3.61	< 0.01
Synthesis and Degradation of Ketone Bodies	4	4	3.66	< 0.01
Mitochondrial fatty acid betaoxidation	9	16	3.16	< 0.01
Cholesterol Biosynthesis	8	15	2.79	< 0.01
Metabotropic glutamate pheromone	6	10	2.78	0.020
Ath + HF diet				
Up-regulated at 6 weeks				
Electron Transport Chain	35	82	4.93	< 0.01
mRNA processing binding Reactome	120	434	3.64	< 0.01
Translation Factors	20	49	3.48	< 0.01
p38 MAPK signaling pathway	13	28	3.36	< 0.01
Unsaturated Fatty Acid Beta Oxidation	4	6	2.78	0.018
Matrix Metalloproteinases	9	24	2.03	0.034
TGF Beta Signaling Pathway	35	124	2.08	0.039
Fas pathway and stress induction	41	149	2.07	0.042
Down-regulated at 6 weeks				
Focal adhesion	56	186	3.51	< 0.01
Steroid Biosynthesis	8	12	4.06	< 0.01
Complement and Coagulation Cascades	20	59	2.70	< 0.01
G Protein Signaling	26	83	2.62	0.013
Calcium regulation in cardiac cells	41	145	2.54	0.014
Cholesterol Biosynthesis	7	15	2.60	0.016
Up-regulated at 24 weeks				
Translation Factors	21	49	3.993	< 0.01
mRNA processing binding Reactome	121	437	4.055	< 0.01
p38 MAPK signaling pathway	12	28	3.016	< 0.01
TGF Beta signaling pathway	35	124	2.077	0.039
Down-regulated at 24 weeks				
Amino Acid Metabolism	19	45	3.891	< 0.01
Urea cycle and metabolism of amino groups	10	20	3.472	< 0.01
Striated muscle contraction	16	42	3.080	< 0.01
Steroid Biosynthesis	6	12	2.689	0.015
Small ligand GPCRs	8	19	2.513	0.020
Glycolysis and Gluconeogenesis	14	41	2.402	0.023

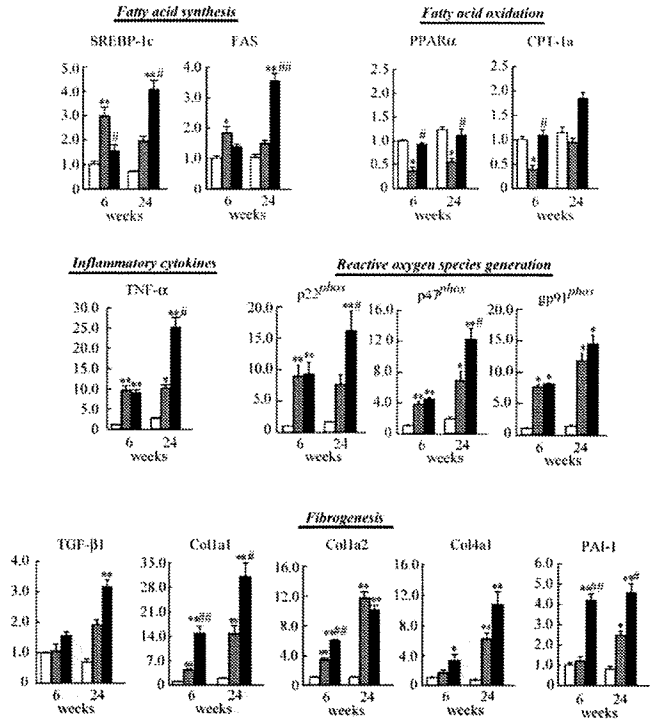

Fig. 6. Quantitative real-time PCR for representative genes involved in steatohepatitis. The mRNA levels of genes for SREBP-1c, FAS, PPARα, CPT-1a, TNF-α, $p22^{phox}$, $p47^{phox}$, $gp91^{phox}$, TGF-β1, procollagen type I alpha 1 (Col1a1), procollagen type I alpha 2 (Col1a2), procollagen type IV alpha 1 (Col4a1), and PAI-1 in the livers of mice fed standard chow (n = 3), the Ath diet (n = 3), or the Ath+HF diet (n = 3) were quantified with real-time PCR after 6 and 24 weeks. The RNA samples used for real-time PCR were the same as those used for the microarray analysis. The gene expression was normalized with eukaryotic 18S ribosomal RNA. The degree of change in the gene expression was based on the mean expression levels of control mice at 6 weeks. The values represent the means ± SEM. *$P < 0.05$ and **$P < 0.01$ versus the control group. #$P < 0.05$ and ##$P < 0.01$ versus the Ath group.

resistance and promotes oxidative stress, the activation of HSCs, and all components of the liver pathology of NASH (steatosis, inflammation, fibrosis, and cellular ballooning); and (4) there is a molecular signature indicative of lipid-induced oxidative stress in the liver, which may play a causal role in the development of steatohepatitis.

To diagnose human NASH, cellular ballooning, in addition to simple steatosis and inflammatory cell infiltration, is one of the most important pathological features.[36] However, ballooning degeneration has scarcely been determined in the existing animal models, including mice fed the MCD diet. We believe that our study is the first to report that cellular ballooning is frequently induced in the livers of mice fed the Ath diet.

Recently, we proved that insulin resistance accelerates the pathological development of steatohepatitis experimentally.[11] In this study, on the basis of the results of the pyruvate challenge test and HOMA-IR, we concluded that the Ath+HF diet causes hepatic insulin resistance. It is known that the excessive accumulation of FFAs caused by the overexpression of lipoprotein lipase[37] and an increase in SREBP-1c–regulated lipogenesis[38] leads to impaired tyrosine phosphorylation of IRS-1 and IRS-2, resulting in hepatic insulin resistance. Furthermore, the up-regulation of SREBP-1c–regulated lipogenesis contributes to the development of insulin resistance via the down-regulation of IRS-2 in the liver.[39,40] Indeed, in our study, the induction of lipoprotein lipase and SREBP-1c and the repression of IRS-2 were detected in the livers of mice fed the Ath diet (Fig. 7). Moreover, the up-regulation of stearoyl–coenzyme A desaturase 1, an enzyme that catalyzes the synthesis of monounsaturated fatty acids, might contribute to lipid accumulation and insulin resistance in the liver, as reported in skeletal muscle.[41] Therefore, the cholesterol-induced and TG-induced alteration of fatty acid metabolism may cause hepatic insulin resistance in this model of steatohepatitis.

Another possible cause of the liver pathology in our model is lipid-induced oxidative stress and its downstream events, as we identified an accumulation of 4-HNE and protein carbonyls, the activation of stellate cells, and hepatic inflammation with cell ballooning. In this study, in addition to cholesterol, the accumulation of TG and FFAs by the addition of a high-fat component accelerated oxidative stress, possibly via the up-regulation of genes involved in the generation of ROS, such as the NADPH oxidase complex, and the down-regulation of genes for antioxidant enzymes. While we were preparing this article, Mari et al.[35] reported that the mitochondrial loading of free cholesterol, but not TG and FFA, decreases mitochondrial glutathione and sensitizes it to the TNF-α–mediated apoptosis of hepatocytes. Therefore, the different kinds of accumulated lipids may cause oxidative stress in the liver additively in different ways. In

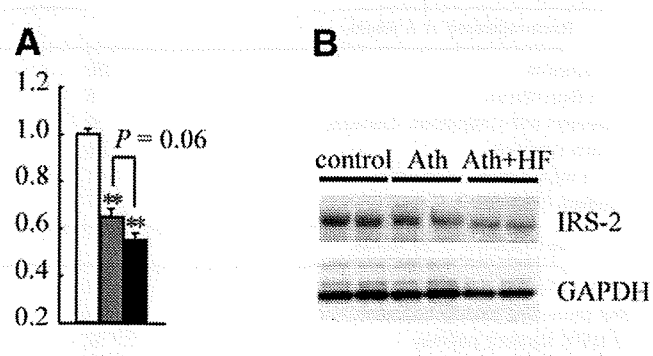

Fig. 7. The Ath and Ath+HF diets decreased the mRNA and protein levels of IRS-2 in the liver. (A) mRNA levels of the IRS-2 genes in the livers of mice fed standard chow (white bar; n = 3), the Ath diet (gray bar; n = 3), or the Ath+HF diet (black bar; n = 3) after 12 weeks. The values represent the means ± the SEM. *$P < 0.05$ versus the control group. #$P < 0.05$ versus the Ath group. (B) Western blot of IRS-2 in the livers of mice fed the standard chow, Ath diet, or Ath+HF diet after 12 weeks.

patients with NASH, impaired glutathione metabolism and antioxidant enzyme activity probably cause an increase in oxidative stress.[42]

The Ath diet induces an Ath lipid profile, including an increase in small dense LDL-C, which is highly susceptible to oxidation, and then leads to oxidized low-density lipoprotein (LDL), which induces an inflammatory response in endothelial cells.[43] In the livers of mice fed the Ath diet, the expression levels of genes for CD36 antigen and scavenger receptor type B member 1, which are receptors for oxidized LDL,[44] tended to be up-regulated (Supplementary Table 2). Therefore, it might be possible that up-regulated receptors for oxidized LDL enhance the uptake of increasing levels of small dense LDL-C and contribute to inflammation in the liver.

In response to the lipid-induced oxidative stress, genes involved in fibrogenesis were coordinately up-regulated. Indeed, the hepatic expression of TNF-α and NADPH oxidase complex genes preceded that of fibrogenic genes, and this suggested that inflammation precedes the fibrogenic process in our models. The expression of TGF-β and PAI-1 genes was up-regulated dramatically, especially in the Ath+HF group. PAI-1 is a key factor in matrix remodeling, and the gene is highly induced in response to TGF-β.[45] Urokinase plasminogen activator generates plasmin, and this process is inhibited by PAI-1. Plasmin degrades the extracellular matrix both directly and by activating matrix metalloproteinases.[46] Therefore, PAI-1 inhibits collagenolysis by inhibiting the generation of plasmin in the liver. Consequently, the inhibition of collagenolysis, in addition to the overall up-regulation of collagen genes, might contribute to hepatic fibrosis in this model.

In summary, we report that the Ath diet induces steatohepatitis with cellular ballooning via cholesterol-induced oxidative stress and hepatic insulin resistance. Adding a high-fat component further aggravates oxidative stress and steatohepatitis, possibly by inducing insulin resistance and down-regulating genes for antioxidant enzymes. This model suggests the critical and different roles of cholesterol, TG, and FFAs in causing oxidative stress and insulin resistance leading to steatohepatitis and provides a system for screening therapeutic targets to treat NASH and atherosclerosis.

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Impact of Diabetes on Recurrence of Hepatocellular Carcinoma after Surgical Treatment in Patients With Viral Hepatitis

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OBJECTIVES: **Consensus has been reached that diabetes is a risk factor for development of HCC, but the impact on postoperative recurrence is still controversial. To clarify this point, we analyzed the relationship of postoperative recurrence rate of HCC and coexistence of diabetes in the patients with viral hepatitis.**

METHODS: **A total of 90 patients who had undergone curative resection for HCC were analyzed. They were divided into two groups with and without diabetes, and the recurrence-free survival rates after surgical treatment and the factors contributing to recurrence were examined.**

RESULTS: **Kaplan-Meier survival analysis showed the recurrence-free survival rates in the diabetic group were significantly lower than those in the nondiabetic group ($P = 0.005$) and overall survival rates in the diabetic group were significantly lower than those in the nondiabetic group ($P = 0.005$). These results were emphasized in the analysis of patients infected with hepatitis C virus. Univariate and multivariate analyses showed diabetes was a significant factor contributing to HCC recurrence after treatment. Furthermore, multivariate analysis in HCC patients with diabetes showed Child-Pugh classification B ($P = 0.001$) and insulin therapy ($P = 0.049$) were significant factors contributing to HCC recurrence after treatment.**

CONCLUSIONS: **The results of the present study suggest that diabetes is a risk factor for the recurrence of HCV-related HCC and decreases the overall survival rates after surgical treatment. HCV-related HCC patients with diabetes should be closely followed for postoperative recurrence.**

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most frequent malignant neoplasm in the world (1), and its prevalence is particularly high in Asia and Africa. The recent increase in its prevalence has attracted the attention of researchers (2, 3). Surgical therapy provides complete cure for HCC, but the indication is limited to a relatively small number of patients. Recent remarkable advances in diagnostic imaging techniques and systematic hepatectomy have been improving the prognosis of patients with HCC, but these techniques have not provided satisfactory results (4, 5), because of a high post-treatment recurrence rate characterizing HCC. Previous studies have noted that factors contributing to recurrence include gender, alcohol consumption, hepatitis C virus (HCV) infection, hepatic reserve, liver fibrosis degree, tumor size, tumor differentiation degree, vascular factor, and alpha-fetoprotein (AFP) level (6–9).

On the other hand, recent studies have reported that coexistence of diabetes is a risk factor for the progression of liver fibrosis and the development of HCC in chronic hepatitis C (10, 11). These reports suggest that coexistence of diabetes is also involved in the high postoperative recurrence rate of HCC. However, it is controversial whether diabetes is an independent risk factor for the post-treatment recurrence of HCC. Ikeda *et al.* (12) reported that diabetes was a risk factor for the recurrence of HCC after surgical treatment, but Poon *et al.* (13) and Toyoda *et al.* (14) reported that this was not the case. The discrepancy among these reports is probably due in part to the difference in the etiology of liver disease in the patients studied.

In this study, to clarify the controversial point about diabetes and HCC recurrence, we examined the impact of diabetes on the postoperative recurrence of HCC in 90 patients who had undergone curative resection for HCC. In addition, we classified the HCC patients into groups of hepatitis B

virus (HBV)- and HCV-related HCC patients, and performed a close analysis of the impact of diabetes on the postoperative recurrence of HCC in each group.

PATIENTS AND METHODS

Patients

A total of 150 patients were diagnosed with primary HCC and underwent surgical treatment in Kanazawa University Hospital between June 1987 and May 2004. Of these patients, 90 were analyzed who had HBV or HCV infection and underwent curative resection.

HCCs were detected by imaging modalities such as ultrasound scan, dynamic CT scan, MR imaging, and abdominal arteriography. The diagnosis of HCC was made by typical hypervascular tumor staining on angiography in addition to using typical findings, which showed hyperattenuation areas in the early phase and hypoattenuation in the late phase on dynamic CT (15).

All resected tumors were examined pathologically for the degree of differentiation of HCC, vascular invasion, and persistence of tumor in the surgical stump. Pathological degree of differentiation of HCC was assessed according to the general rules for the clinical and pathologic study of primary liver cancer (16).

Treatment and Follow-Up

In selecting surgery as a treatment option for HCC, we considered the following criteria: (a) good general condition of the patient whose Karnofsky performance status was over 80, (b) primary HCC, (c) Child-Pugh classification A or B, (d) the number of HCC was solitary and no CT, MRI, or angiographic evidence of vascular invasion or distant metastasis. Curative resection was defined as complete excision of the tumor with tumor-free surgical margins and no local recurrence at the surgical margin within 6 months after surgery.

Patients were followed postoperatively on an outpatient basis by abdominal ultrasound, dynamic CT, or MRI at 3-month intervals for at least 60 months. Recurrence was diagnosed by dynamic CT or MRI, and the date of recurrence was defined as the date of examination when the recurrence of HCC was noted. In patients with recurrent HCC, the recurrence-free period was defined as the time between the date of surgery and the date of recurrence. We confirmed the date of the patient's last visit to our hospital and checked the status of HCC using each patient's medical record.

Laboratory and Virologic Testing

Blood samples were tested for hepatitis B surface antigen (HBs-Ag) and hepatitis C virus antibody (HCV-Ab) by commercial immunoassays (Fuji Rebio, Tokyo, Japan). Serum AFP level was measured by enzyme immunoassay (AxSYM AFP, Abbott Japan, Tokyo, Japan). Diabetes was diagnosed according to the American Diabetes Association criteria for type II diabetes (17) and the severity of liver disease (stage of fibrosis) was evaluated according to the criteria of Desmet *et al.* (18).

Statistical Analysis

Between-group differences were assessed by univariate analysis with Student's *t*-test for numerical data and the $\chi 2$ test with Yates' correction (or Fisher's exact test where appropriate) for nominal data. Overall survival and recurrence-free survival was examined using the method of Kaplan-Meier, and differences were assessed by the log-rank test. Impact factors for the recurrence of HCC after hepatic resection were analyzed by univariate and multivariate analysis using Cox proportional hazards model. Seventeen variables were analyzed, consisting of age, gender, etiology, body mass index (BMI), prevalence of alcohol abuse, diabetes, hemoglobin A1c (HbA1c), liver fibrosis degree, Child-Pugh classification, platelet count, alanine aminotranseferase (ALT), T-bil, Alb, AFP, tumor size, tumor differentiation degree, and the presence of vascular invasion. $P < 0.05$ was considered statistically significant.

RESULTS

Comparison of Baseline Characteristics

Of the 90 patients (75 men and 15 women, with a mean age of 61.0 yr) who were followed and analyzed, 30 were diagnosed as having coexistence of diabetes, and 60 had no diabetes. The characteristics of the patients in both groups

Table 1. Characteristics of Patients

Characteristic	Patients With Diabetes (N = 30)	Patients Without Diabetes (N = 60)	*P* Value
Median age (yr)	62.0	60.6	0.453
Gender (male/female)	24/6	51/9	0.560
Etiology (HBV/HCV/ HBV + HCV)	8/22/0	17/40/3	0.438
Body mass index (kg/m^2)	23.5	22.73	0.316
Alcohol abuse (+/−)	13/17	27/33	0.881
HbA1c (%)	6.4	4.8	<0.001
HOMA-IR	4.1	3.3	0.399
Platelet count ($\times 10^4/\mu$L)	12.2	13.5	0.284
ALT (IU/L)	69.8	56.4	0.318
Total bilirubin (mg/dL)	0.9	0.8	0.510
Albumin (g/dL)	4.1	4.2	0.341
AFP (ng/mL)	417	395	0.931
Fibrosis (F1/F2/F3/F4)	0/4/4/22	4/5/5/46	0.732
Inflammatory grading (A1/A2/A3)	11/17/2	24/33/3	0.385
Child-Pugh grade (A/B/C)	24/6/0	53/7/0	0.294
Tumor size (mm)	34.3	29.8	0.359
Diff. degree (wel/mod/por)*	11/12/7	20/19/21	0.264
Vascular invasion (+/−)	11/19	20/40	0.757
Date of operation (1987–1995/1995–2000/ 2001–2004)	8/14/8	13/30/17	0.538

*Histological degree of HCC: wel = well differentiated; mod = moderately differentiated; por = poorly differentiated.

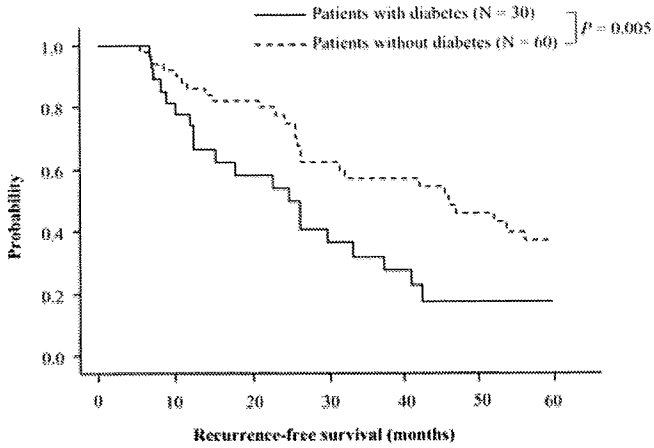

Figure 1. Kaplan-Meier curves for recurrence-free survival in the groups of patients with and without diabetes.

are shown in Table 1. No significant differences were noted between the two groups in age, gender, HBV or HCV infection rate, BMI, or prevalence of alcohol abuse. HbA1c was significantly higher, at 6.4%, in the diabetic group than in the nondiabetic group with an HbA1c of 4.8% ($P < 0.001$). There were no significant differences in platelet count, ALT, T-bil, Alb, AFP, or Child-Pugh classification between the two groups. In addition, no significant differences were observed in the liver fibrosis degree, or in the size, degree of differentiation, and presence of microscopic vascular invasion of resected HCC. Homeostasis model assessment-insulin resistance (HOMA-IR) of the patients with diabetes, which was high compared with that of Japanese healthy subjects (19), was higher than that of the patients without diabetes, although it was not statistically significant.

Impact of Diabetes on Recurrence After Surgical Treatment of HCC

Next, the diabetic and nondiabetic groups were compared for the rate of HCC recurrence after surgical treatment. HCC recurred after surgical treatment in 49 patients, consisting of 22 diabetic patients (73.3%) and 27 nondiabetic patients (45.0%). The mean time to recurrence was 32.8 months (range 8–60 months) and the median time to recurrence was 29.4 months.

Figure 1 shows the Kaplan-Meier curves for recurrence-free survival of the patients with and without diabetes. The recurrence-free survival rates 1, 2, 3, 4, and 5 yr after surgical treatment were 77.8%, 55.6%, 36.0%, 16.7%, and 16.7%, respectively, in the diabetic patient group, and 89.5%, 80.4%, 56.8%, 45.0%, and 36.6%, respectively, in the nondiabetic patient group; all rates except 1 yr were significantly lower in the diabetic patient group ($P = 0.155$, $P = 0.010$, $P = 0.009$, $P = 0.002$, and $P = 0.005$).

To further examine the degree of contribution of diabetes to the postoperative recurrence of HCC, we performed univariate and multivariate analysis. Univariate analysis identified the following variables as factors significantly contributing to HCC recurrence after surgical treatment: presence of diabetes ($P = 0.003$), high HbA1c level ($P < 0.001$), and Child-Pugh classification B against A ($P < 0.001$) (Table 2). When we conducted multivariate analysis, we chose variables that had already pointed out a risk factor for HCC recurrence and the P value was lower than 0.1 in univariate analysis. As a result, multivariate analysis of these variables showed that the presence of diabetes (risk 2.9, 95% CI 1.5–5.4, $P < 0.001$) and Child-Pugh classification B against A (risk 3.6, 95% CI 1.7–7.7, $P = 0.001$) were significant factors contributing to HCC recurrence after surgical treatment (Table 3).

Table 2. Univariate Proportional Hazard Model for Recurrence of HCC After Surgical Treatment

Variable	Hazard Ratio	95% CI	P Value
Age (yr)	1.0	1.0–1.1	0.258
Gender (male)	0.5	0.3–1.1	0.104
Etiology (HCV)	1.0	0.5–1.8	0.965
Body mass index (>25 kg/m^2)	1.2	0.6–2.3	0.554
Alcohol abuse (+)	1.1	0.7–2.0	0.644
Diabetes (+)	2.4	1.3–4.2	0.003
HbA1c (%)	1.5	1.2–1.9	<0.001
Fibrosis (F4)	1.4	0.7–3.1	0.349
Child-Pugh grade (B)	3.1	1.6–6.0	<0.001
Platelet count ($\times 10^4/\mu$L)	1.0	0.9–1.0	0.465
ALT (IU/L)	1.0	1.0–1.1	0.717
Total bilirubin (mg/dL)	1.4	0.6–3.6	0.451
Albumin (g/dL)	1.4	0.8–2.4	0.225
AFP (>200 ng/mL)	1.0	0.5–1.8	0.906
Tumor size (>50 mm)	1.5	0.7–1.2	0.213
Diff. degree (P)	1.1	0.6–2.1	0.675
Vascular invasion (+)	1.2	0.4–1.7	0.490

Impact of Diabetes on Prognosis After Surgical Treatment of HCC

To examine the impact of diabetes on prognosis of HCC patients, we analyzed the overall survival rates after surgical treatment. Figure 2 shows the Kaplan-Meier curves for overall survival of the patients with and without diabetes after surgical treatment of HCC. The overall survival rates 1, 2, 3, 4, and 5 yr after surgical treatment were 100%, 88.9%, 75.0%, 63.6%, and 45.5%, respectively, in the diabetic patient group, and 100%, 98.1%, 88.9%, 85.7%, and 76.3%, respectively, in the nondiabetic patient group; the rates of more than 3 yr were significantly lower in the diabetic patient group ($P = 1.000$, $P = 0.073$, $P = 0.028$, $P = 0.039$, and $P = 0.005$).

Table 3. Multivariate Proportional Hazard Model for Recurrence of HCC After Surgical Treatment

Variable	Hazard Ratio	95% CI	P Value
Diabetes	2.9	1.5–5.5	<0.001
Fibrosis (F4)	1.9	0.8–4.5	0.148
Child-Pugh grade (B)	3.6	1.7–7.7	0.001
AFP(>200 ng/mL)	0.7	0.3–1.5	0.390
Diff. degree (P)	0.9	0.5–1.8	0.776
Vascular invasion (+)	2.0	1.0–4.0	0.061

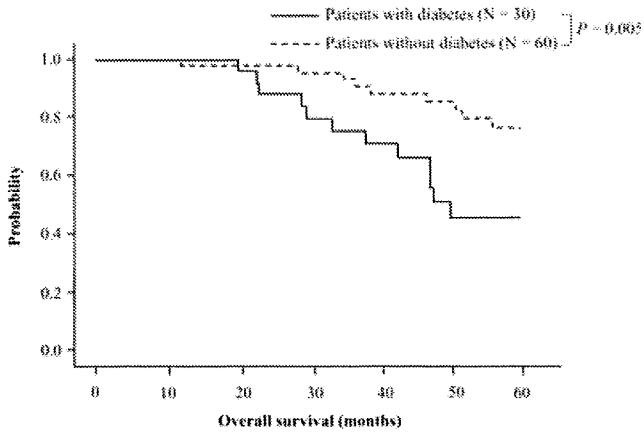

Figure 2. Kaplan-Meier curves for overall survival in the groups of patients with and without diabetes.

These curves indicated that overall survival rates were significantly lower in the diabetic patient group ($P = 0.005$).

Differential Impact of Diabetes on Prognosis After Surgical Treatment Between HBV- and HCV-Infected Patients

Next, we classified the HCC patients into HBV- and HCV-related HCC patients, and examined the impact of diabetes on recurrence-free and overall survival rates after surgical treatment. We divided all patients into 25 HBs-Ag (+), HCV-Ab (−) patients (with HBV-related HCC) and 62 HBs-Ag (−), HCV-Ab (+) patients (with HCV-related HCC), and further divided these two groups of patients into four groups according to the presence or absence of diabetes. In 62 patients who were HCV-Ab positive, 53 patients were also positive for HCVRNA. The other 9 patients were not examined for HCVRNA. The clinical profiles of these four groups of patients are shown in Table 4. Three HBs-Ag (+), HCV-Ab (+) patients, who were not complicated by diabetes, were excluded from the analysis. There were no significant differences between the groups of HBV-related HCC patients with and without diabetes in age, gender, BMI, prevalence of alcohol abuse, platelet count, ALT, total bilirubin, Child-Pugh classification, liver fibrosis degree, tumor size, tumor differentiation degree, or the presence of vascular invasion, except for Alb and AFP. Similarly, there were no significant differences between the groups of HCV-related HCC patients with and without diabetes. The HbA1c levels were higher in the groups of diabetic patients with HBV- or HCV-related HCC.

The Kaplan-Meier curves for recurrence-free survival in the groups of HBV-related HCC patients with and without diabetes are shown in Figure 3. The recurrence-free survival rates 1, 3, and 5 yr after surgical treatment were 85.7%, 57.1%, and 42.9%, respectively, in the diabetic patient group, and 76.5%, 46.7%, and 40.0%, respectively, in the nondiabetic patient group, showing no significant differences between the two groups ($P = 0.596$, $P = 0.670$, and $P = 0.827$). In the analysis of overall survival in the groups of HBV-related HCC patients with and without diabetes by the method of Kaplan-Meier, it indicated that there was no difference between the two groups ($P = 0.505$) (Fig. 4).

Figure 5 shows the Kaplan-Meier curves for recurrence-free survival in the groups of HCV-related HCC patients with and without diabetes. The recurrence-free survival rates 1, 2, 3, 4, and 5 yr after surgical treatment were 75.0%, 38.9%, 22.2%, 11.1%, and 11.1%, respectively, in the diabetic patient group, and 94.6%, 83.9%, 62.1%, 35.0%, and 29.2%, respectively, in the nondiabetic patient group, indicating that the recurrence-free survival rates were significantly lower in the diabetic patient group than in the nondiabetic patient group ($P = 0.030$, $P < 0.001$, $P < 0.001$, $P < 0.001$, and $P < 0.001$).

In the analysis of overall survival in the groups of HCV-related HCC patients with and without diabetes, the overall survival rates 1, 2, 3, 4, and 5 yr after surgical treatment were 100%, 88.9%, 76.5%, 64.7%, and 43.8%, respectively, in the diabetic patient group, and 100%, 100%, 96.7%, 92.5%, and

Table 4. Characteristics of Patients With HBV- or HCV-Related HCC

	Patients With HBV-Related HCC			Patients With HCV-Related HCC		
Characteristic	With Diabetes (N = 8)	Without Diabetes (N = 17)	*P* Value	With Diabetes (N = 22)	Without Diabetes (N = 40)	*P* Value
Median age (yr)	61.6	57.3	0.3	62.2	62.9	0.737
Gender (male/female)	7/1	13/4	0.5	17/5	35/5	0.302
Body mass index (kg/m^2)	23.4	23.2	0.9	23.5	22.7	0.348
Alcohol abuse (+/−)	2/6	6/11	0.6	11/11	21/19	0.853
HbA1c (%)	5.9	4.6	0.07	6.6	4.9	<0.001
Fibrosis (F1/F2/F3/F4)	0/0/2/6	3/2/0/12	0.8	0/4/2/16	1/3/5/31	0.680
Child-Pugh grade (A/B)	6/2/0	15/2	0.4	18/4	35/5	0.551
Platelet count ($\times 10^4/\mu$L)	11.5	13.9	0.3	12.4	13.5	0.520
ALT (IU/L)	31.8	42.3	0.6	84.2	63.5	0.233
Total bilirubin (mg/dL)	0.9	0.9	1.0	0.9	0.8	0.303
Albumin (g/dL)	3.8	4.2	0.02	4.2	4.2	0.983
AFP (ng/mL)	1,056	121	0.001	162	328	0.460
Tumor size (mm)	28.9	31.2	0.8	36.0	29.6	0.295
Diff.degree (W/M/P)	2/2/4	5/7/5	0.3	9/10/3	14/11/15	0.058
Vascular invasion (+/−)	1/7	4/13	0.5	12/10	15/25	0.548

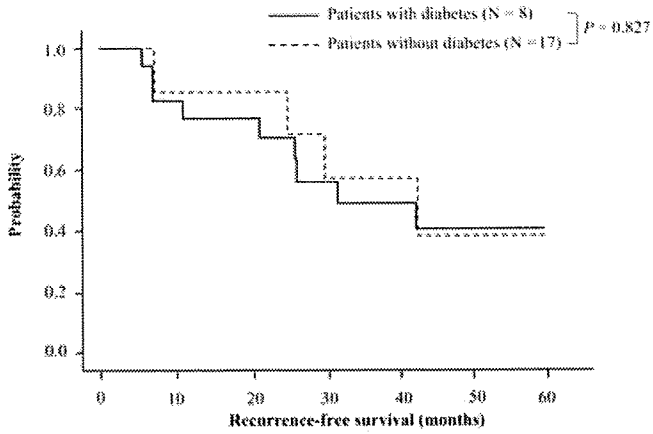

Figure 3. Kaplan-Meier curves for recurrence-free survival in HBV patients with diabetes and HBV patients without diabetes.

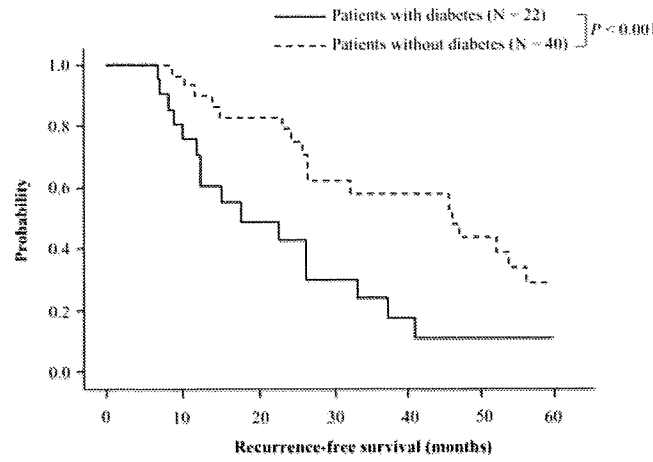

Figure 5. Kaplan-Meier curves for recurrence-free survival in HCV patients with diabetes and HCV patients without diabetes.

82.6%, respectively, in the nondiabetic patient group, indicating that the overall survival rates of more than 3 yr were significantly lower than in the nondiabetic patient group ($P = 1.000$, $P = 1.000$, $P = 0.035$, $P = 0.015$, $P = 0.004$) (Fig. 6).

Factors Associated With Recurrence-Free Survival After Surgical Treatment for HCC in Patients With Diabetes

Finally, we performed univariate and multivariate analyses to determine the variables that might affect the postoperative recurrence of HCC in the 30 HCC patients with diabetes, consisting of 17, 4, and 9 patients receiving insulin therapy, oral hypoglycemic drugs, and no treatment, respectively. Univariate analysis identified Child-Pugh classification B as a factor significantly contributing to the postoperative recurrence of HCC ($P < 0.001$) (Table 5). When we conducted multivariate analysis, we chose variables that had been already pointed out as a risk factor for HCC recurrence and whose P value was lower than 0.1 in univariate analysis. Multivariate analysis identified Child-Pugh classification B (risk 40.0, 95% CI 4.4–362.1, $P = 0.001$) and the presence of insulin therapy (risk 3.9, 95% CI 1.0–15.3, $P = 0.049$) as factors significantly contributing to the postoperative recurrence of HCC (Table 6). In multivariate analysis, both factors showed significant P value. Based on the results, we considered that both factors contribute to recurrence of HCC independently.

DISCUSSION

In the present study, univariate and multivariate analyses identified the presence of diabetes as a factor significantly contributing to the recurrence of HCC after surgical treatment. The results are consistent with the findings of Ikeda *et al.* (12). They analyzed a population of 64 HBV-related HCC patients and a larger population of 144 HCV-related HCC patients, but did not compare the postoperative recurrence rate between the two populations. In our study, 25 and 62 patients with HBV- and HCV-related HCC, respectively, were included, similar to the proportion of such patients in the study by Ikeda *et al.* (12), presumably leading to similar results. On the other hand, none of the variables that have been reported to contribute to the postoperative recurrence of HCC, such as liver fibrosis degree, Alb level, AFP level, tumor differentiation degree,

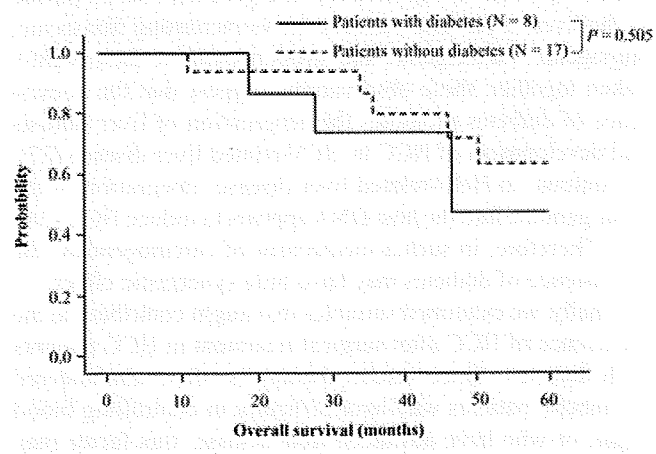

Figure 4. Kaplan-Meier curves for overall survival in HBV patients with diabetes and HBV patients without diabetes.

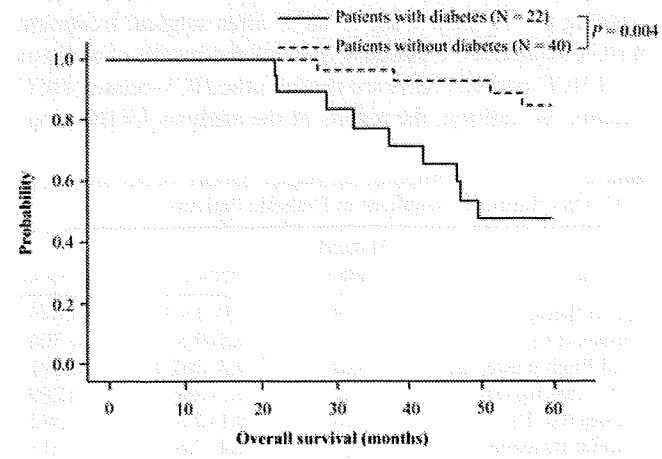

Figure 6. Kaplan-Meier curves for overall survival in HCV patients with diabetes and HCV patients without diabetes.

Table 5. Univariate Proportional Hazard Model for Recurrence of HCC After Surgical Treatment in Diabetic Patients

Variable	Hazard Ratio	95% CI	*P* Value
Age (yr)	1.0	1.0–1.1	0.534
Gender (male)	0.8	0.3–2.2	0.681
Etiology (HCV)	0.5	0.2–1.4	0.183
Body mass index (>25 kg/m^2)	1.0	0.4–2.6	0.938
Alcohol abuse (+)	1.0	0.4–2.3	0.972
HbA1c (%)	1.4	1.0–1.9	0.059
Fibrosis (F4)	1.2	0.5–3.3	0.651
Child-Pugh grade (A/B)	11.8	3.2–43.9	<0.001
Platelet count ($\times 10^4/\mu$L)	1.0	0.9-1.1	0.548
ALT(IU/L)	1.0	1.0–1.0	0.699
Total bilirubin (mg/dL)	0.6	0.2–2.5	0.506
Albumin (g/dL)	1.2	0.5–2.9	0.689
AFP (>200 ng/dL)	0.9	0.4–2.3	0.899
Tumor size (>50 mm)	0.8	0.7-1.2	0.668
Diff degree (W/M/P)	0.9	0.4–2.0	0.723
Vascular invasion	0.9	0.4–2.2	0.843
Insulin therapy	2.5	1.0–6.6	0.058

and the presence of vascular invasion, were identified as significant factors. This is probably because of our criteria for surgical treatment and that patients who recurred within 6 months after surgery were excluded from the study, resulting in the inclusion of only a population with little variation in these variables.

Next, groups of patients with HBV- and HCV-related HCC were separately examined for the impact of diabetes on the recurrence of HCC after surgical treatment. No significant differences in the recurrence-free survival rates determined by the Kaplan-Meier curve were noted between the HBV-related HCC patient groups with and without diabetes, which was similar to the results reported by Poon *et al.* (13), Toyoda *et al.* (14), and Huo *et al.* (20). In contrast, the recurrence-free survival rate was significantly lower in the group of HCV-related HCC patients with diabetes than in the group of HCV-related HCC patients without diabetes. From the above findings, we concluded that the coexistence of diabetes was a factor contributing to the recurrence of HCC after surgical treatment in HCV-related HCC patients, and that the results of analysis of all HCC patients reflected those in the HCV-related HCC patients. In addition, the results of the analysis for the prognosis of HCV-related HCC patients after surgical treatment showed that the overall survival rate was significantly lower in the diabetic patient group than in the nondiabetic group. These results suggest that more frequent recurrence may contribute to shorter survival in HCV-related HCC patients with diabetes.

Table 6. Multivariate Proportional Hazard Model for Recurrence of HCC After Surgical Treatment in Diabetic Patients

Variable	Hazard Ratio	95% CI	*P* Value
Insulin therapy (+)	3.9	1.0–15.3	0.049
Fibrosis (F4)	2.2	0.5–9.8	0.306
Child-Pugh grade (B)	40.0	4.4–362.1	0.001
AFP (>200 ng/mL)	2.1	0.5–8.8	0.289
Diff degree (P)	0.6	0.1–2.8	0.542
Vascular invasion (+)	1.7	0.4–7.6	0.513
Etiology (HCV)	2.0	0.3–12.2	0.460
HbA1c (%)	1.1	0.8–1.6	0.629

To our knowledge, only one study has examined the impact of diabetes on the recurrence of HCC after surgical treatment separately in HBV- and HCV-related HCC patients. Contrary to the results of this study, Huo *et al.* (20) have reported that diabetes is not a risk factor for the recurrence of HCV-related HCC. The clinical characteristics of HCC patients, such as the number of tumors, tumor diameter, and background liver histology, differed between their study and ours, and the presence or absence of vascular invasion and hepatic reserve indicated by Child-Pugh classification were unknown in their study, which makes direct comparison difficult, but partially accounts for the different results. Although, to date, no studies have reported that, as shown in this study, there is a possibility that diabetes differently affects the postoperative recurrence of HCC in the groups of patients with HBV- or HCV-related HCC.

This may be because of different mechanisms of carcinogenesis in the two groups (21). It appears that neither HBV nor HCV damages liver cells, but these viruses induce chronic inflammation in the liver, and facilitate mutations in liver cells, leading to their malignant transformation (22, 23). Our previous study using the microarray technique showed that the genes expressed in the liver differed markedly between HBV- and HCV-related liver disease patients (24). This genetic heterogeneity is considered to be associated with different modes of pathogenesis of HBV- and HCV-related HCC (25–28). Previous studies have shown that, in HCV-related HCC, chronic inflammation and oxidative stress are closely associated with hepatocellular death and regeneration (29–33). Highly insulin-resistant diabetics show increased peripheral lipolysis and hepatic accumulation of free fatty acids (34, 35). The β-oxidation of fatty acids in mitochondria is decreased in these patients, and they are under high oxidative stress. We also previously reported that the gene expression profile in the liver of diabetic patients shows increasing fibrogenic, angiogenic, tumorigenic, and stress responsive factors (36). Taken together, these observations suggest that the coexistence of diabetes promotes the progression of liver fibrosis and development of HCC in HCV-related liver disease (37). In contrast, in HBV-related liver disease, integration of the virus genome into the host DNA appears to induce HCC (38–40). Therefore, in such a mechanism of carcinogenesis, the coexistence of diabetes may have little synergistic effect.

Finally, we examined variables that might contribute to the recurrence of HCC after surgical treatment in HCC patients with diabetes. Since insulin therapy is often administered to diabetic patients who have difficulty in controlling blood sugar, or who have advanced liver disease, this factor may be involved in the recurrence of HCC in those under insulin therapy. Therefore, we included HbA1c, liver fibrosis degree,

and Child-Pugh classification together with insulin therapy in multivariate analysis. As a result, multivariate analysis identified Child-Pugh classification B and insulin therapy as significant factors contributing to the postoperative recurrence of HCC. These findings suggest that insulin therapy and Child-Pugh classification B are independent risk factors for postoperative recurrence.

The mechanism by which insulin promotes HCC recurrence is unknown. However, the results of this study are consistent with the report that insulin acts as a tumor growth factor *in vitro* (41). In animal models, insulin has been shown to be a promoter of colonic carcinogenesis (42). Although there has been much debate about the use of insulin and the risk of cancer development, no consensus has been reached (43–45). A recent study has indicated that insulin therapy is a risk factor for the postoperative recurrence of colorectal cancer (46). These findings show the possibility that insulin therapy promotes HCC recurrence after surgical treatment. It should be discussed how to use insulin therapy in HCC patients with diabetes in the future.

There is a limitation to our study, because our study is retrospective and on a not so large population. However, the results of the present study suggest that diabetes is a risk factor for the recurrence of HCV-related HCC and decreases the overall survival rates after surgical treatment. HCV-related HCC patients with diabetes should be closely followed for post-treatment recurrence, and blood sugar control may also be important to reduce the rate of recurrence. However, since the use of insulin to treat diabetes in HCC patients may promote tumor recurrence, treatment methods for blood sugar control require further evaluation.

STUDY HIGHLIGHTS

What Is Current Knowledge

- Diabetes accumulates liver fibrosis for chronic hepatitis C.
- Diabetes is a risk factor for hepatocellular carcinoma (HCC).
- HCC has high recurrence rate after curative surgery.

What Is New Here

- Hepatitis C virus (HCV) related patients with diabetes have a higher possibility of HCC recurrence.
- HCV-related patients with diabetes have poorer prognosis.
- Controlling of blood sugar may reduce HCC recurrence.
- Insulin therapy may accumulate HCC recurrence.

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CONFLICT OF INTEREST

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CORRESPONDENCE

External Validation of FIB-4: Diagnostic Accuracy Is Limited in Elderly Populations

To the Editor:

We read with interest the articles by Stering et al.[1] and Vallet-Pichard et al.[2] The former authors developed the FIB-4 index, a noninvasive method for assessing liver fibrosis in patients with HIV/HCV coinfection. The variables used are age, aspartate aminotransferase (AST), alanine aminotransferase (ALT) and platelet (PLT) count, and the formula is as follows: (age [yr] × AST [U/L])/ ((PLT[10^9/L]) × (ALT[U/L])$^{1/2}$). They showed that over 70% of patients could be classified into either absence or presence of advanced fibrosis by cutoff of <1.45 or >3.25 respectively, with diagnostic accuracy of 87%. The latter authors expanded the applicability of the FIB-4 index to HCV-monoinfected patients and showed that 73% of patients were classified with diagnostic accuracy of 93%, an excellent performance in both classification and accuracy of diagnosis.

Because the mean age of patients was young in these studies (40 years[1] and 44 years[2]), we wondered whether this index could also fit to Japanese patients who are rather older than the Western patients. We validated the FIB-4 index in a retrospective cohort of 1,405 patients who underwent liver biopsy at our hospital. The mean age was 55 ± 12 years. The distribution of METAVIR fibrosis scores was as follows: 1.6% showed no fibrosis (F0), 44.8% showed mild fibrosis (F1), 29.5% showed moderate fibrosis (F2), 20.2% showed severe fibrosis (F3), and 3.9% showed cirrhosis (F4). The proportion of advanced fibrosis (F3 or F4) was slightly higher in our population compared to the former studies (24.1% vs. 20.7% [1] and 17.2% [2]). As shown in Table 1, only 53% of patients were classified to either <1.45 or >3.25, a much lower rate than previous reports. The diagnostic accuracy was excellent in patients with a FIB-4 index <1.45 (94%), however, it was relatively poor in patients with a FIB-4 index >3.25 (50%) making the overall accuracy as low as 67%.

We supposed this discordance with previous reports may be derived from the older age of our populations and thus we categorized patients into three groups according to age and analyzed separately. In patients with age ≤50 years, 64% of patients were classified, and the diagnostic accuracy was 94% for a FIB-4 index <1.45 and 68% for a FIB-4 index >3.25 making the overall accuracy of 90%, a result comparable to previous reports. In older patients, however, diagnostic accuracy was significantly low compared to those with age ≤50 years (56% for age 51-60 years, $P < 0.0001$ and 51% for age ≥60 years, $P < 0.0001$). Because patients with a FIB-4 index >3.25 increased according to age (6%, 34%, and 53% for ages ≤50, 51-60 and >60 years), and the diagnostic accuracy was low in these patients (48% to 50%), these results suggest that, in elderly patients, a variable "age" generates excessively high FIB-4 index leading to misclassification of no-moderate fibrosis (F0-F2) into a FIB-4 index >3.25.

In conclusion, the FIB-4 index could accurately differentiate advanced fibrosis in young Japanese patients with chronic hepatitis C but the diagnostic accuracy is limited in the elderly. Thus, in elderly patients, some sort of adjustment for the effect of age on FIB-4 index may be necessary for more precise classification.

Table 1. Comparison of FIB-4 Index and Liver Biopsy Results in Terms of Age

	METAVIR Fibrosis Score				Diagnostic Accuracy
	FIB-4	F0-2	F3-4	Total	
All patients					
	<1.45	283 (20%)	18 (1%)	301 (21%)	94%
	>3.25	228 (16%)	226 (16%)	454 (32%)	50%
	1.45-3.25	556 (40%)	94 (7%)	650 (47%)	
	Total	1067 (76%)	338 (24%)	1405 (100%)	67%
Age ≤50	<1.45	240 (54%)	16 (4%)	256 (58%)	94%
(Mean 40 yrs)	>3.25	9 (2%)	19 (4%)	28 (6%)	68%
	1.45-3.25	126 (28%)	38 (8%)	164 (36%)	
	Total	375 (84%)	73 (16%)	448 (100%)	90%
Age 51-60	<1.45	30 (7%)	2 (1%)	32 (8%)	94%
(Mean 56 yrs)	>3.25	76 (18%)	69 (16%)	145 (34%)	48%
	1.45-3.25	215 (50%)	36 (8%)	251 (58%)	
	Total	321 (75%)	107 (25%)	428 (100%)	56%
Age >60	<1.45	13 (2%)	0 (0%)	13 (2%)	100%
(Mean 66 yrs)	>3.25	143 (27%)	138 (26%)	281 (53%)	49%
	1.45-3.25	215 (41%)	20 (4%)	235 (45%)	
	Total	371 (70%)	158 (30%)	529 (100%)	51%

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Reply:

We thank Kurosaki and colleagues for interest in our article that developed a simple noninvasive index, FIB-4, to accurately assess fibrosis in patients coinfected with hepatitis C virus (HCV) and human immunodeficiency virus (HIV).[1] This model was recently validated in a large cohort of patients monoinfected with HCV, and had similar accuracy and was found to be as good as or better than other noninvasive models that use nonroutine tests.[2] In these 2 studies, the mean ages were 40 and 44 years, respectively. Therefore, the utility of this index in older patients is not known.

The current report in a large cohort of Japanese patients with HCV found that although FIB-4 had excellent accuracy (90%) in those < 50 years of age, it did not perform well in those > 50 (56% in those 51-60 years of age and 51% in those > 60 years old) despite an increasing proportion of advanced fibrosis in the older patients (25%-30% versus 16% in those < 50). The reason for the drop in performance of FIB-4 in older patients in the present study is not clear but may be due in part to patient demographics. Although the overall proportion of patients with advanced fibrosis were similar in the 3 cohorts, the proportion of patients with cirrhosis in the Japanese cohort was only 3.9% compared to 7.2% in the study by Vallet-Pichard[2] and 15% in our coinfected population.[1] Furthermore, we are not told the distribution of F4 cirrhosis among the 3 age groups in the current study, which could have affected their results.

Whenever an index includes both a numerator and denominator, changes in either can affect the results. Age was included in the numerator due to the observation that increasing age is associated with increasing fibrosis.[3] This is supported in the current analysis, which found increasing advanced fibrosis in those > 50 years of age. Another

Risk for hepatocellular carcinoma with respect to hepatitis B virus genotypes B/C, specific mutations of enhancer II/core promoter/precore regions and HBV DNA levels

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ABSTRACT

Background/aim: To examine the risks for hepatocellular carcinoma (HCC) with respect to hepatitis B virus (HBV) genotypes, specific viral mutations (MT), serum HBV DNA levels, and cirrhosis.

Methods: HBV genotypes, 1653/1753/core promoter (CP)/precore MT and HBV DNA levels were determined in 248 HBV patients with HCC and 248 HBV controls.

Results: Genotype C, CP-MT, T1653, HBV DNA levels ≥ 4 $\log_{10}$ copies/ml and cirrhosis had a higher risk for HCC compared to patients with genotype B ($p = 0.001$, OR 1.9), CP wild-type (WT) ($p<0.001$, OR 4.1), C1653 ($p = 0.028$, OR 2.4), HBV DNA <4 $\log_{10}$ copies/ml ($p = 0.003$, OR 2.1) and without cirrhosis ($p<0.001$, OR 4.0) respectively. Multivariate analysis showed that CP-MT, T1653, HBV DNA ≥ 4 $\log_{10}$ copies/ml and cirrhosis were independent factors for HCC (all $p<0.05$). A receiver operating characteristics curve showed no cut-off HBV DNA level associated with minimal chance of HCC. Patients with CP-MT and cirrhosis had a 22.2-fold increased risk of HCC compared to patients with CP-WT and without cirrhosis. Patients with CP-MT and HBV DNA levels ≥ 4 $\log_{10}$ copies/ml had a 7.2-fold increased risk of HCC compared to patients with CP-WT and HBV DNA levels <4 $\log_{10}$ copies/ml. Patients with CP-MT and T1653 had a 9.9-fold increased risk of HCC compared to patients with wild-type for both regions.

Conclusions: CP-MT, T1653, HBV DNA levels ≥ 4 $\log_{10}$ copies/ml and cirrhosis are independent factors for development of HCC. The risks increased substantially in patients having these factors in combination.

Hepatocellular carcinoma (HCC) is a disease of global concern, occurring in over 20% of the 400 million people with chronic hepatitis B infection (CHB). While the exact mechanisms of hepatocarcinogenesis with CHB infection remain elusive, several virological factors have been identified to be possibly associated with a higher risk of development of HCC. These include hepatitis B virus load (HBV DNA) levels, HBV genotypes, core promoter and precore mutations. These factors are also associated with the development of cirrhosis and its complications.[1 2]

The majority of the published studies examining HBV genotypes compare genotypes B and C in relation to the disease profile of CHB because these are the two main genotypes prevailing in Asia, a region contributing around 75% of the world's population of CHB. However, while some studies suggest genotype C has a higher risk of development of HCC,[3–5] this observation is not substantiated by others.[6–8] One large study conducted in Taiwan shows that genotype B is more commonly found in patients with HCC developed at a young age.[9] In the Caucasian and Indian populations, genotype D is associated with a greater risk for HCC than genotype A.

Concerning the common naturally occurring mutations at the precore (G1896A) and core promoter (A1762T and G1764A) regions, some studies show that patients with precore mutants have more aggressive disease including reactivation of CHB and fulminating course of the disease,[10 11] These observations have not been substantiated in other studies partly because the predominant genotypes are different between Asia and Europe/USA.[12 13] For core promoter mutations, some studies report a higher risk of development of HCC in patients with core promoter mutations compared to those with wild-type.[3 6 7 14–16] Again, this has not been confirmed by other studies.[5 17] In addition to these two common mutations, two other mutations, C to T at 1653 in the enhancer II region and T to C/A/G (V) at 1753 in the core promoter region, have recently been found to be associated with the development of HCC.[18–20]

The uncertainty as to whether these virological factors are genuine risk factors for the development of HCC may be due to several reasons. Most of the studies only have a limited number of patients. These studies often examine only specific virological factors; for example, genotypes without considering the possible confounding effect of other parameters, such as viral mutations and HBV DNA levels. Indeed the associations between genotype B with precore mutations and genotype C with core promoter mutations have been shown to be confounding factors.[21] Whether there are any additive or synergistic effects on the risks of HCC development with different combinations of genotypes/precore/core promoter and mutations in the enhancer II region and HBV DNA levels have not been studied. Finally, the risks for development of HCC of these factors in the setting of cirrhosis have not been examined.

Therefore we sought to examine the risks of HBV DNA levels, HBV genotypes, core promoter/precore/T1653/V1753 mutations and cirrhosis individually and in combination for the development of HCC in a large population study.

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PATIENTS AND METHODS

Patients

A total of consecutive 248 Chinese CHB patients with HCC were recruited from Department of Medicine and Department of Surgery, The University of Hong Kong, Queen Mary Hospital, Hong Kong from 2000 to 2004. All patients had the diagnosis of HCC for the first time, during regular follow-up in our centre ($n = 198$) or in other hospitals ($n = 50$). Patients with recurrent HCC were excluded from the present study. One hundred and twenty patients had histologically proven HCC. The remaining 128 patients had elevated α-fetoprotein (AFP) with typical imaging features in computerised tomography and/or magnetic resonance imaging and/or hepatic angiogram.

During the same period of recruitment of patients with HCC, 4825 CHB Chinese patients without HCC were being followed up in the University Liver Clinic of Queen Mary Hospital, Hong Kong. A consecutive 248 CHB patients without HCC were recruited as controls. These control patients were matched individually with each patient with HCC for gender, age (less than 2 years difference) and hepatitis B e antigen (HBeAg)/antibody to HBeAg (anti-HBe) status in a 1:1 ratio. The absence of HCC was assured by the absence of any space occupying lesion by ultrasonography performed on two separate occasions 1 year apart.

All patients were positive for hepatitis B surface antigen (HBsAg) checked by radioimmunoassay (AUSRIA II, Abbott Laboratories, North Chicago, IL) for at least 6 months. HBeAg/anti-HBe was also determined by the same assay. Patients with other concomitant diseases including hepatitis C or D virus infection, autoimmune hepatitis, Wilson's disease, primary biliary cirrhosis, alcoholic liver disease and fatty liver (diagnosed by ultrasonography) were excluded.

Liver cirrhosis is defined by the score of >2 according to the aspartate aminotransferase (AST) to platelet ratio index (APRI) calculated from the following formula: ([AST/upper limit of normal]/platelet count [$\times 10^9$/litre]) $\times$ 100.[22]

Methods

Stored serum at −70°C were thawed for the determination of the HBV DNA levels, HBV genotypes, core promoter and precore mutations and finally the mutations at the enhancer II region. The HBV DNA levels were measured by Cobas Amplicor HBV Monitor test (Roche Diagnostics, Branchburg, NJ) with a lower limit of detection of 300 copies/ml.

HBV genotypes were determined by the enzyme linked immunosorbent assay (ELISA). The detailed methodology of the assay was described in our previous study.[23] The sequence of core promoter and precore regions including A1762T/G1764A (core promoter mutations) and G1896A (precore mutation) were determined by direct sequencing. The methodology was described in our previous study.[20] The two recently identified HCC-related mutations at the enhancer II and core promoter regions namely, C to T at 1653 and T to C/A/G (V) at 1753, were also sequenced according to the methods described in our previous study[20] in 140 patients with HCC and 100 control patients with adequate sera available for sequencing. There were no differences in the median age (range), male to female and HBeAg: anti-HBe ratios between these two subgroups of 140 and 100 patients [56.6 years (29–83.7) vs. 59.8 years (24.8–81.6), p = 0.13 for age; 114:26 vs. 79: 21, p = 0.65 for male to female ratio; and 40:100 vs. 23:77, p = 0.28 for HBeAg: anti-HBe ratio).

Statistical analysis

All statistical analyses were performed using the SPSS 14.0 for Windows, SPSS Inc., Chicago, IL). The Mann–Whitney U test was used to compare continuous variables between patients with HCC and control patients. The χ^2 test with Yates correction factor or Fisher's exact test was used to compare categorical variables between two groups. A receiver operating characteristic (ROC) curve was used to determine whether there is a cut-off HBV DNA which was associated with no risk of HCC. Logistic regression was adopted to determine independent risk factors for HCC. The adjusted odds ratios (OR) for development of HCC of different combinations of variables were also calculated by the logistic regression analysis with a selected combination defined as the reference. All estimates were accompanied by a 95% confidence interval (CI), where appropriate and a p-value <0.05 was considered as statistical significance.

RESULTS

Demographics

The demographic data for 248 patients with HCC and 248 control patients are listed in table 1. Patients with HCC had a significantly poorer liver biochemical parameters and higher median AFP level compared to control patients. Patients with HCC also had a higher prevalence of liver cirrhosis compared to control patients. The OR for patients with cirrhosis was 4.0 [95% CI, 2.8 to 5.9].

HBV genotypes

A total of 478 out of 496 (96.2%) samples had positive genotype results from EIA test, but this test gave indeterminate results for the remaining 18 samples (10 from patients with HCC, eight from control patients). Of the 238 patients with HCC with genotype results, 67 (28.2%) had genotypes B, 170 (71.4%) had genotypes C and one (0.4%) had genotype D. Of the 240 control patients with genotype results, 100 (41.7%) had genotypes B, 135 (56.3%) had genotype C, three (1.3%) had genotypes D and two (0.8%) had mixed genotypes.

Comparing patients with either genotypes B or C, patients with HCC had a higher prevalence of genotype C compared to control patients [170/237 (71.2%) vs. 135/235 (57.4%) respectively; p = 0.001; OR 1.9; 95% CI, 1.3 to 2.8].

Core promoter and precore mutations

Of all the 496 samples, direct sequencing failed to generate results for 70 samples for core promoter region and 61 samples for precore region.

Table 1 Demographic data for the study population

	Patients with HCC (n = 248)	Control patients (n = 248)
Sex (M:F)	199:49	199:49
Age (years)	57.5 (24.8–83.7)	57.7 (24.8–81.8)
HBeAg:anti-HBe (%)	61:187 (24.6%:75.4%)	61:187 (24.6%:75.4%)
Albumin (g/l)	37 (16–59)*	43 (17–53)*
Bilirubin (μmol/l)	17 (5–531)†	12 (2–96)†
ALT (U/l)	57 (4–1154)‡	46 (9–920)‡
AFP (ng/ml)	136.5 (1–1 060 000)§	5 (1–200)§
Presence of cirrhosis (%)	170 (68.5%)¶	87 (35.1%)¶

*,†,‡,§,¶p<0.001.
Continuous variables are expressed in median (range).
ALT, alanine aminotransferase; AFF, α-fetoprotein.

Results of core promoter mutations were successfully obtained in 194 (78.2%) samples of patients with HCC and in 232 (93.5%) samples of control patients. Patients with HCC had a higher prevalence of core promoter mutations compared to patients without HCC [173/194 (89.2%) vs. 155/232 (66.8%), respectively; p<0.001; OR, 4.1 (95% CI, 2.4 to 6.9)].

Results of precore mutations were successfully obtained by the direct sequencing in 198 (79.8%) samples of patients with HCC and in 237 (95.6%) samples of patients without HCC. There was no significant difference in the prevalence of precore mutations between patients with and without HCC [72/198 (36.4%) vs. 106/237 (44.7%), respectively; p = 0.10].

Relationship between HBV genotypes and core promoter/precore mutations

Patients with genotype B had a higher chance of harbouring precore mutations compared to patients with genotype C [105/144 (72.9%) vs. 67/267 (25.1%), respectively; p<0.001; OR, 8.0; 95% CI, 5.1 to 12.7)]. Patients with genotype C had a higher chance of harbouring core promoter mutations compared to patients with genotype B [237/264 (89.8%) vs. 76/141 (53.9%), respectively; p<0.001; OR, 7.5; 95% CI, 4.5 to 12.6].

HBV DNA levels

To determine whether there is an exact HBV DNA level below which HCC is unlikely to occur, the HBV DNA levels of all the patients with or without HCC were entered in the ROC curve analysis (fig. 1). The ROC nearly overlapped with reference line with the area under the curve (AUC) of 0.51 (p = 0.75; 95% CI, 0.46 to 0.56) indicating that there existed no cut-off HBV DNA level that was associated with minimal risk of HCC. Further separate analysis of patients who had HBeAg seroconversion (anti-HBe positive) with less fluctuation of HBV DNA levels during the course of the disease was performed. The AUC was only 0.56 (p = 0.054; 95% CI, 0.50 to 0.62). This suboptimal value confirmed that there was no HBV DNA level that was associated with minimal risk of HCC even for anti-HBe-positive patients.

Though a "safe" lower limit of HBV DNA level could not be identified, a higher proportion of patients with HCC had high viral load defined by HBV DNA level ≥4 $\log_{10}$ copies/ml compared to that of control patients [218/248 (87.9%) vs. 193/248 (77.8%), respectively; p = 0.003; OR, 2.1; 95% CI, 1.3 to 3.4].

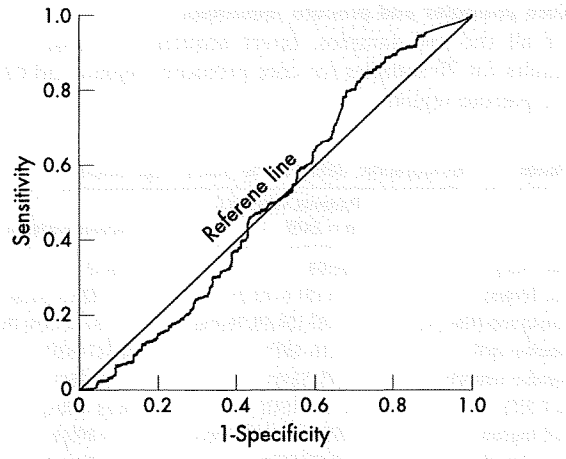

Figure 1 Receiver operating characteristics curve (ROC) of HBV DNA levels and development of HCC (area under curve = 0.51) (p = 0.75; 95% CI, 0.46 to 0.56).

T1653 and V1753 mutations

Results of T1653 and V1753 were successfully obtained by the direct sequencing in 133 (95%) out of 140 samples of patients with HCC and in 99 out of 100 (99%) samples of control patients. The reason for samples with no obtainable results for these two mutations was due to the failure of generation of sequence with good quality by direct sequencing. Patients with HCC had a significantly higher prevalence of T1653 mutations compared to control patients [19.5% (26/133) vs. 9.1% (9/99), respectively; p = 0.028; OR, 2.4; 95% CI, 1.1 to 5.4]. There was no difference in the prevalence of T1753 mutations between patients with HCC and control patients [42.1% (56/133) vs. 44.4% (44/99), respectively; p = 0.72].

Multivariate analysis on the risk factors for HCC

HBV genotypes, core promoter mutations, T1653 mutations, HBV DNA levels and presence of cirrhosis were entered into the logistic regression analysis. Core promoter mutations, T1653 mutation, HBV DNA levels ≥4 $\log_{10}$ copies/ml and presence of cirrhosis were shown to be independent factors associated with HCC (p = 0.015, 0.044, 0.048 and 0.005, respectively). Genotype C, identified as a significant risk factor in the univariate analysis was *not* an independent risk factor for HCC.

Relationship between core promoter mutations, T1653 mutations, HBV DNA levels and cirrhosis

A higher proportion of patients with core promoter mutations had high viral load (HBV DNA ≥4 $\log_{10}$ copies/ml) compared to that of patients without core promoter mutations [284/328 (86.6%) vs. 71/98 (72.4%), respectively; p = 0.001; OR, 2.6; 95% CI, 1.4 to 4.2]. There was no difference in the prevalence of T1653 mutation between patients with core promoter mutations and wild-type [28/174 (16.1%) vs. 6/43 (14.0%), respectively; p = 0.91]. Patients with core promoter mutations had a significantly higher prevalence of cirrhosis compared to patients with core promoter wild-type [177/328 (54.0%) vs. 39/98 (39.8%), respectively; p = 0.014; OR, 1.8; 95% CI, 1.1 to 2.8].

Adjusted risks for patients with core promoter mutations stratified according to HBV DNA levels, 1653 mutations and cirrhosis

Stratifying core promoter mutations, 1653 mutations, HBV DNA levels and presence of cirrhosis to assess the combined risk for the development of HCC resulted in 16 different groups of patients with certain groups having fewer than five patients, thus precluding reliable statistical analysis. Therefore separate analyses were performed by stratifying (1) core promoter mutations according to HBV DNA levels, (2) core promoter mutations with or without concomitant 1653 mutations and (3) core promoter mutations according to presence or absence of cirrhosis. The adjusted odds ratios for the development of HCC are shown in tables 2, 3 and 4, respectively.

DISCUSSION

To our knowledge, the present study is the largest study examining the individual role as well as the possible interacting effects of HBV genotypes, the two commonly occurring mutations (core promoter and precore mutations), mutations at the enhancer II (T1653) and at the more upstream core promoter region (V1753), HBV DNA levels, and liver cirrhosis

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Table 2 Adjusted odds ratios for HCC in patients with core promoter wild-type/mutations according to the HBV DNA levels

Core promoter	HBV DNA (log_{10} copies/ml)	Number of patients	Odds ratio (95% CI)	p Value
Wild-type	<4	27	Reference	–
Wild-type	≥4	77	1.8 (0.6 to 6.0)	0.33
Mutant	<4	44	3.1 (0.9 to 10.6)	0.07
Mutant	≥4	426	7.2 (2.4 to 21.4)	<0.001

CI, confidence interval.

on the development of HCC. This relatively large number of patients would allow any possible links or associations between these factors contributing to the development of HCC to be defined more unequivocally. One of the limitations of the present study is that the role of deletions in the pre-S region of HBV genome which have been recently shown to be associated with the development of HCC has not been studied.[24]

An epiphenomenon observed in the present study was the higher risk of HCC in patients with genotype C compared to patients with genotype B (all were subgenotype B2 in our locality according to our previous study).[25] This is apparently consistent with other studies.[3–5] However, genotype C was not found to be an independent factor for HCC when tested in the multivariate analysis. Core promoter mutations, T1653 mutations, high HBV DNA levels and presence of cirrhosis were independent risk factors for HCC. This is not an unusual finding because of the strong association of genotype C with core promoter mutations (89.8%), and genotype B with precore mutations (72.9%). Though it is well proven that patients with genotype B have an earlier HBeAg seroconversion,[21 26] it appears neither genotype B nor C has any major influential effects on the life-time risk of HCC, a finding in concordance with other studies.[6 27] We have recently shown that the earlier HBeAg seroconversion with genotype B is related to the more intense immunogenic stimulation during the immunoclearance phase.[28] The effects exerted by HBV genotypes B and C on the disease progression of CHB subsequent to HBeAg seroconversion appear to be similar.

However, there are at least two documented effects accompanying core promoter mutations on the development of HCC. Mutations in the core promoter region result in a shift change of the viral pregenomic secondary structure which may enhance the viral replication.[29] Viral replication can also be further enhanced by a second mechanism in which the transcription of the pregenomic RNA will be increased through the removal of the nuclear receptor binding site and creation of a hepatocytes nuclear binding factor.[30] These changes increase the core RNA transcription with enhanced core protein, DNA polymerase, pre-genomic RNA synthesis, but suppress the precore RNA transcription whose normal function is to decrease pregenomic RNA packaging.[31 32] This is in complete concordance with the finding of the present study and of Chauhan and colleagues.[33] HBV DNA levels were higher in patients with core promoter mutations compared to those without core promoter mutations.

Table 3 Adjusted odds ratios for HCC in patients with core promoter wild-type/mutations according mutations at 1653

Core promoter	1653	Number of patients	Odds ratio (95% CI)	p Value
Wild-type	Wild-type	37	Reference	–
Wild-type	Mutant	6	2.7 (0.5 to 15.6)	0.27
Mutant	Wild-type	146	3.6 (1.6 to 7.9)	0.02
Mutant	Mutant	28	9.9 (3.1 to 31.5)	<0.001

CI, confidence interval.

Table 4 Adjusted odds ratios for HCC in patients with core promoter wild-type/mutations according presence or absence of cirrhosis

Core promoter	Cirrhosis	Number of patients	Odds ratio (95% CI)	p Value
Wild-type	No	59	Reference	–
Wild-type	Yes	39	7.5 (2.5 to 23.0)	<0.001
Mutant	No	151	6.0 (2.3 to 15.9)	<0.001
Mutant	Yes	177	22.2 (8.4 to 58.4)	<0.001

CI, confidence interval.

In the present study, by setting the patients without core promoter mutations and HBV DNA <4 log_{10} copies/ml as a reference, the adjusted odds ratio for HCC for patients with core promoter mutations at the same viraemic level was 3.1 (95% CI, 0.9 to 10.6), with a borderline p value of 0.07 (table 2). It is possible that the higher risk of HCC in patients with core promoter mutations may also be mediated through another additional pathway independent of the increase in viral replication. The possible carcinogenic mechanisms require further *in vitro* studies and functional analyses to delineate.

The present study demonstrated that the risk of HCC was substantially increased in patients harbouring core promoter mutations and having liver cirrhosis, a 22.2-fold increase when compared to patients with core promoter wild-type and without cirrhosis (table 4). Similarly, patients with core promoter mutations with high HBV DNA levels of ≥4 log_{10} copies/ml had a 7.2-fold increase risk of HCC when compared to patients with core promoter wild-type with HBV DNA levels <4 log_{10} copies/ml (table 2).

In the present study, we found that T1653 was an independent risk factor for the development of HCC. According to our previous studies,[20 34] T1653 mutation is associated with HCC in patients with genotype C. In the present study, we further confirmed with larger number of patients that T1653 was an independent risk factor for HCC irrespective of HBV genotypes. 1653 is located in the box alpha of the enhancer II region of HBV genome. The C to T mutation at 1653 converts histidine to tyrosine at amino acid 94 of the X protein which may explain its association with the hepatocarcinogenesis. According to Takahashi and colleagues, the frequency of T1653 mutation increases with the progression of liver disease from chronic hepatitis to cirrhosis.[19] It occurs later than, and independent of, core promoter mutations in chronic hepatitis B disease. However, when both viral mutations, that is, core promoter and T1653 mutations, co-existed, the risk of HCC was substantially increased to 9.9-fold when compared to patients with wild-type at both genomic regions (table 3).

Finally, the present study showed that there was no reliable cut-off HBV DNA level associated with low risk of HCC. This means that maximal viral suppression to the lowest possible HBV DNA levels should be the target for future management of CHB disease.

In conclusion, core promoter mutations, T1653 mutations, HBV DNA levels ≥4 log_{10} copies/ml and presence of cirrhosis were independent factors for the development of HCC. The risk increased substantially in patients who carried these factors in combination. Future studies should consider these factors in conjunction with age and gender of patients to formulate the risk of HCC in CHB patients.

Competing interests: None.

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BASIC—LIVER, PANCREAS, AND BILIARY TRACT

Direct Cytopathic Effects of Particular Hepatitis B Virus Genotypes in Severe Combined Immunodeficiency Transgenic With Urokinase-Type Plasminogen Activator Mouse With Human Hepatocytes

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Background & Aims: **Little is known about the direct cytopathic effect of hepatitis B virus (HBV) and its association with particular viral genotypes or genetic mutations. We investigate HBV genotype–related differences in viral replication, antigen expression, and histopathology in severe combined immunodeficiency transgenic with urokinase-type plasminogen activator mice harboring human hepatocytes.** ***Methods:*** **Mice were inoculated with wild-type of different genotype strains (3 for each HBV/A2, B1, and C2) recovered from preinfected-mice sera or patient sera.** ***Results:*** **Histologic analysis of mice infected with HBV/C2 for 22–25 weeks showed abundant ground-glass appearance of the hepatocytes and fibrosis in the humanized part of the murine liver owing to the activation of hepatic stellate cells mediated by oxidative stress through transforming growth factor-β1 signaling, whereas neither was observed with HBV/A2 and B1. The HBV-DNA level in sera was the highest in mice infected with HBV/C2 compared with those with HBV/A2 and HBV/B1 (10^9, 10^7, and 10^4 log copies/mL, respectively, $P < .05$) during 6–8 weeks postinoculation. HB core-related antigen excretion had a similar trend among the genotypes, whereas secretion of HB surface antigen was more pronounced for HBV/A2 followed by HBV/C2 and much less for HBV/B1. Introduction of precore stop-codon mutation in the HBV/B1 caused a significant increase in viral replication, antigen expression, and a histopathologic picture similar to HBV/C2.** ***Conclusions:*** **By using a humanized in vivo model, we show that different HBV genotypes and even particular mutations resulted in different virologic and histopathologic outcomes of infection, indicating that particular genetic variants of HBV may be directly cytopathic in immunosuppressive conditions.**

With an estimated 420 million chronic carriers, hepatitis B virus (HBV) infection is one of the most prevalent chronic viral infections of human beings. The chronic infection often leads to cirrhosis and/or hepatocellular carcinoma, which is responsible for at least 1 million deaths annually worldwide.[1] The precise mechanism by which chronic viral hepatitis results in hepatocellular carcinoma (HCC) is not known. However, evidence now is available concerning the direct effects of HBV in this process.[2,3] The important issue of a distinct impact of the various HBV genotypes on the virulence has not been addressed directly so far.[4,5]

Genotypes are subdivided further into subgenotypes on the basis of phylogenetic relationships.[6] Evidence for the influence of HBV genotypes/subgenotypes on liver diseases in acute, fulminant, and chronic infection have been reported increasingly.[7-13] Involvement of genetic mutations of HBV in its pathogenesis is another open question. Previous reports have indicated that mutations in basal core promoter, precore/core, envelope, and X coding regions may be associated with HCC.[14] The term *precore mutants* refers to HBV strains with nonsense frameshift or initiation codon mutation in the precore region that prevent translation of hepatitis B e antigen (HBeAg) precursor and are associated with an increase of viral replication via stabilization of the pregenomic encapsidation signal.[15] However, little is known about the histopathologic implication of the mutants. Complexity

Abbreviations used in this paper: α-SMA, α-smooth muscle actin; PCm, precore stop-codon mutation; HBcrAg, antigens related to hepatitis B virus core; HSC, hepatic stellate cell; 8-OHdG, 8-hydroxydeoxyguanosine; PCR, polymerase chain reaction; ROS, reactive oxygen species; TGF-β1, transforming growth factor-β1.

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