Table 2 Hematological adverse events among patients with advanced biliary tract cancer treated with gemcitabine/S-1 combination chemotherapy ($n = 25$)

	Grade 1	Grade 2	Grade 3	Grade 4	Incidence of grade 3–4 events (%)
Neutropenia	0	5	12	2	56
Leukopenia	1	9	6	0	24
Anemia	5	7	1	1	8
Thrombocytopenia	1	4	1	0	4
Febrile neutropenia	–	–	0	0	0

Table 3 Non-hematological adverse events among patients with advanced biliary tract cancer treated with gemcitabine/S-1 combination chemotherapy ($n = 25$)

	Grade 1	Grade 2	Grade 3	Grade 4	Incidence of grade 3–4 events (%)	Incidence of grade 1–4 events (%)
Fatigue	8	3	2	0	8	52
Anorexia	3	2	2	0	8	28
Diarrhea	1	4	1	0	4	24
Constipation	1	6	0	0	0	28
Rash	9	3	0	0	0	48
Fever	8	3	0	0	0	44
Hand-foot rash	7	3	0	–	0	40
Infection-other	8	2	0	0	0	40
Nausea	3	2	0	0	0	20
Stomatitis	5	1	0	0	0	24
Allergic reaction	4	1	0	0	0	20
Hyperpigmentation	8	0	–	–	0	32
Alopecia	3	0	–	–	0	12
Injection site reaction	2	0	0	–	0	8
Vomiting	1	0	0	0	0	4
Hyperbilirubinemia	3	1	4	0	16	32
AST	11	5	0	0	0	64
ALT	8	4	2	0	8	56
Creatinine	3	0	0	0	0	12

AST aspartate aminotransferase, *ALT* alanine aminotransferase

non-hematological adverse events were fatigue (8%), anorexia (8%) and diarrhea (4%).

Discussion

In our population of patients with advanced biliary tract cancer, gemcitabine/S-1 combination chemotherapy achieved an MST of 12.7 months and a 1-year survival rate of 52%. The MST for patients with gall bladder cancer ($n = 8$) was shorter (7.6 months) than that for patients with other cancer types (16.0 months), which is consistent with the findings of previous studies and possibly reflects the more aggressive nature of gall bladder cancer [8, 10, 20]. The proportion of patients with gall bladder cancer in our study (32%) was comparable with the proportions in previous randomized trials (26–39%) [6, 25, 26], so the good MST observed in the current study was unlikely to be simply due to tumor-type selection bias. Furthermore, this was a multi-institution trial, and the eligibility criteria were almost identical to the indications used for administering chemotherapy in daily clinical practice; both these factors are likely to have contributed to reducing selection bias. Although comparing single-arm phase II studies can be problematic, our current results are comparable to those of Sasaki et al., who observed an MST of 11.6 months and a 1-year survival rate of 44% among patients with advanced biliary tract cancer treated with gemcitabine/S-1 combination chemotherapy [20] (Table 4). Their treatment schedule differed slightly from ours: it consisted of 1,000 mg/m^2 gemcitabine on days 1 and 15 and 80 mg/m^2 S-1 daily for 14 consecutive days every 4 weeks. In this study, grade 3–4 neutropenia was observed in 56% of patients, and this often caused suspension of chemotherapy on day 8. In fact, planned chemotherapy administration on day 8 needed to be suspended in 28.5% of cycles. Meanwhile, Sasaki et al.

Table 4 Results of clinical trials of gemcitabine and oral fluoropyrimidine combination chemotherapy for the treatment of advanced biliary tract cancer

	Present study	Sasaki et al. [20]	Knox et al. [10]	Cho et al. [2]	Koeberle et al. [11]
Oral fluoropyrimidine	S-1	S-1	Capecitabine	Capecitabine	Capecitabine
MST (months)	12.7	11.6	14	14	13.2
1-year survival rate (%)	52	44	49	58	N/A
Prevalence of gall bladder cancer (%)	32	40	49	16	18
Incidence of grade 3–4 neutropenia (%)	56	34	34	11	11[a]
Incidence of grade 3–4 anorexia (%)	8	3	N/A	2	7
Incidence of grade 3–4 fatigue (%)	8	N/A	4	0	11
Sample size	25	35	45	44	44

MST median survival time, *N/A* not available

[a] These subjects had leukopenia

reported grade 3–4 neutropenia was 34%, and their regimen might have an advantage of avoiding suspension of chemotherapy due to neutropenia because gemcitabine administration was scheduled on day 1 and 15, not on day 8 with 2-week interval. Interestingly, a previous study of gemcitabine/cisplatin combination therapy in Japanese patients (BT-22 study) yielded a 56.1% incidence rate of grade 3–4 neutropenia, whereas in ABC-02 study involving the same regimen, the rate was only 22.6% among Caucasian patients [6, 26]. Although we need to take into account the difference of treatment duration between 2 studies (up to 24 weeks in ABC-02 study versus up to 48 weeks in BT-22 study), it is tempting to speculate that ethnic differences exist between patients with biliary tract cancer in terms of susceptibility to gemcitabine-related neutropenia. In spite of the high incidence of grade 3–4 neutropenia in the present study, no patients developed febrile neutropenia, probably due to the short duration of neutropenia caused by this combination therapy. Aside from AST/ALT elevation, the most common non-hematological toxicity was fatigue (52%); however, the incidence rates of grade 3–4 toxicities were relatively low, showing that this regimen was generally well tolerated in an outpatient setting. The grade 3–4 hyperbilirubinemia observed in this study was associated with obstructive jaundice caused by the primary disease and so was unlikely to be relevant to the combination therapy regimen. In vitro study also demonstrated the advantage of gemcitabine/S-1 combination. Yoshizawa et al. tested the combination of S-1 with other anticancer drugs (gemcitabine, cisplatin, irinotecan, mitomycin C, adriamycin and paclitaxel) and reported that synergic effect was most evident in gemcitabine/S-1 combination [30]. The combination of gemcitabine and another oral fluoropyrimidine, capecitabine, was found to be similarly efficacious in previous single-arm phase II studies [2, 10, 11]. In their respective studies, Cho et al. [2] observed an MST of 14 months and a 1-year survival rate of 58%, and Knox et al. [10] also observed an MST of 14 months and a 1-year survival rate of 49%. Koeberle et al. [11] found similar results, with an MST of 13.2 months (Table 4). Koeberle et al. also highlighted the importance of maintaining a balance between treatment efficacy and quality of life in palliative chemotherapy for advanced biliary tract cancer. From the point of view of quality of life, combination therapy using oral fluoropyrimidines has the major advantage of being very convenient to administer. Clearly, we must be cautious about the interpretation of data from single-arm phase II studies; however, the combination of gemcitabine and oral fluoropyrimidines can be used for patients with advanced biliary tract cancer in situations that preclude the use of cisplatin (e.g., allergy to cisplatin). In summary, gemcitabine/S-1 combination chemotherapy yielded a promising survival benefit with acceptable toxicity in patients with advanced biliary tract cancer. We believe that this regimen would be a good candidate for the experimental arm of a future phase III trial of gemcitabine/cisplatin combination therapy.

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Case Report

De novo hepatitis B virus infection in hepatocellular carcinoma following eradication of hepatitis C virus by interferon therapy

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Epidemiological studies have revealed that hepatocellular carcinoma (HCC) is still observed in hepatitis C virus (HCV)-positive patients with a sustained response to interferon (IFN) treatment, although a substantial decrease in the incidence of hepatocellular carcinoma (HCC) has been achieved in those patients. Why HCC develops in patients who have a complete clearance of HCV remains unclear. Here, we provided evidence of latent hepatitis B virus (HBV) infection in an initially HCV-positive chronic hepatitis patient who developed HCC after the complete eradication of HCV by IFN therapy. Although he was initially negative for anti-hepatitis B surface antigen (HBsAg) or circulating HBV DNA but positive for anti-hepatitis B core antigen (anti-HBc) in his sera, he developed HBsAg and HBV DNA during the course of the management of a series of cancers. HBV DNA was detectable in the liver tissues before HBV reactivation and the viral sequences derived from his anti-HBc-positive liver showed 100% homology to that from the serum after HBsAg appearance. These findings indicates that HCV-positive individuals who are positive for anti-HBc in the absence of HBsAg could have latent HBV infection in their liver tissues and intrahepatic HBV infection may play a pivotal role in the development of HCC after the IFN-mediated eradication of HCV.

Key words: hepatitis B virus, hepatitis C virus, hepatocellular carcinoma, interferon

INTRODUCTION

DE NOVO HEPATITIS B virus (HBV) infection is defined as the latent viral infection in individuals that lack any of the serological markers for HBV antigens such as anti-hepatitis B surface antigen (HBsAg).[1] In most cases, however, anti-hepatitis B core antigen (anti-HBc) is frequently detectable and, thus, positivity for anti-HBc is believed to be a surrogate marker for *de novo* HBV infection. Although the clinical significance of *de novo* HBV infection has long been an object of study, direct evidence of actual HBV infection in the liver tissue of patients with positive for anti-HBc but negative for HBsAg is lacking.

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The transmission of HBV to transplant recipients via liver grafts gave the first evidence of the latent HBV infection in HBsAg-negative but anti-HBc-positive individuals.[2] We have demonstrated that the majority of healthy individuals positive for anti-HBc were latently infected by the episomal form of HBV with ongoing viral replication, and could be the high risk source of HBV transmission to the living donor-related liver transplant recipients.[3] In agreement with our report, several studies revealed the HBV transmission from anti-HBc-positive donors via liver transplant.[4,5] In contrast to the *de novo* HBV infection after liver transplantation, the actual prevalence of latent HBV infection in anti-HBc-positive patients with hepatitis C virus (HCV)-related chronic liver disease remains controversial. However, numbers of studies have reported that HBV DNA is frequently detected in liver tumors in anti-HBc-positive, HBsAg-negative patients with HCV-related chronic liver disease.[6–9] In addition, anti-HBc is detectable in approximately 50% of patients with HCV-related chronic liver disease and the proportion of anti-HBc-positive patients

increased in association with the progression of liver disease.[10] Notably, our recent prospective observation study on 872 patients with chronic HCV infection demonstrated that anti-HBc-positive results on serological testing are a marker of high risk for hepatocellular carcinoma (HCC) in patients with HCV-related cirrhosis.[11] These findings suggested that prevalence of occult HBV infection is substantially high among patients with HCV-related chronic liver disease and the latently infected HBV might be involved in the development of HCC. *De novo* HBV infection is therefore of particular concern in this subset of patients, since it might provide the clue of the pathogenesis of HCV-related HCC. To support the possibility that latently infected HBV is indeed present in the liver of patients with chronic HCV infection and could play a role on hepatocarcinogenesis, we reported here the reactivation of latently infected HBV in an initially HCV-positive chronic hepatitis patient who developed HCC after eradication of HCV by interferon (IFN) therapy.

CASE REPORT

A 69-YEAR-OLD MAN attended our hospital in September 1992 for chronic liver dysfunction that had been detected during a regular checkup at his workplace. He showed an elevated level of alanine aminotransferase (107 U/L), was positive for HCV infection, and was diagnosed with chronic HCV (Table S1). He lacked any risk factors for HCC development, as evidenced by the absence of alcoholism and smoking, history of diabetes mellitus, or obesity. In February 1993, he began receiving IFN therapy (subcutaneous 6×10^6 units of IFN-α-2b, three times per week) every 24 weeks. In February 1994, we confirmed the sustained disappearance of serum HCV RNA by qualitative polymerase chain reaction (PCR) assay after the cessation of antiviral therapy. His blood tests showed normal liver function.

Five years after the clearance of HCV infection, abdominal ultrasonography and enhanced-computed tomography detected two hepatic tumors, 3.6 cm and 2.0 cm in diameter. Although his serum α-fetoprotein concentration was within the normal limit at that time, we suspected that these lesions were HCC and a right lobectomy was performed in October 1998.

Histological observation revealed that both tumors were moderately differentiated HCC with a trabecular pattern at stage II (T2N0M0), while the surrounding nontumorous liver tissues showed no signs of liver inflammation or other abnormalities. The next year, he was diagnosed with advanced colorectal cancer in the sigmoid colon. He received a sigmoidectomy and histological findings revealed that the tumor was moderately differentiated colorectal cancers at stage IIa (T3N0M0).

At the time of HCC diagnosis he was initially positive for anti-HBc and anti-HBs, but negative for HBsAg and HBV DNA in his serum. Immunohistochemical evaluation showed no hepatitis viral activities in the liver samples. During the management of a series of cancers, his serological level of HBsAg began to increase spontaneously and the serum HBV DNA and HBsAg became positive in June 2004. Although HBV DNA was not detectable initially in his serum by nested-PCR, it was detectable in the tumor and the surrounding nontumorous tissues of the liver specimens, suggesting that he had been latently infected with HBV in the liver tissues at the time of the operation for HCC. Before the appearance of HBsAg in his serum, he had never received blood product transfusion and he lacked any other risk factors for viral transmission such as intravenous drug injection or high risk of sexual transmission (Figure 1).

To confirm that the HBV appearing in his serum was derived from the viruses initially infecting his liver tissues, we employed sequencing analysis to compare the nucleotide sequences of viral strains in the serum

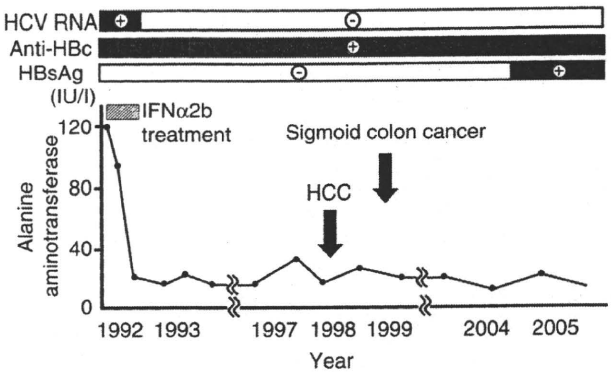

Figure 1 Clinical course of *de novo* hepatitis B virus (HBV) reactivation in the hepatocellular carcinoma (HCC) patient who was initially given a diagnosis of chronic hepatitis C virus (HCV). The patient initially had HCV-related chronic hepatitis with serological markers positive for anti-hepatitis B core antigen but negative for hepatitis B surface antigen (HBsAg), and developed HCC after the complete eradication of HCV infection by interferon therapy. Although he initially lacked the evidence of circulating HBV in his sera, he developed the appearance of HBsAg and HBV DNA during the course of the management of cancers.

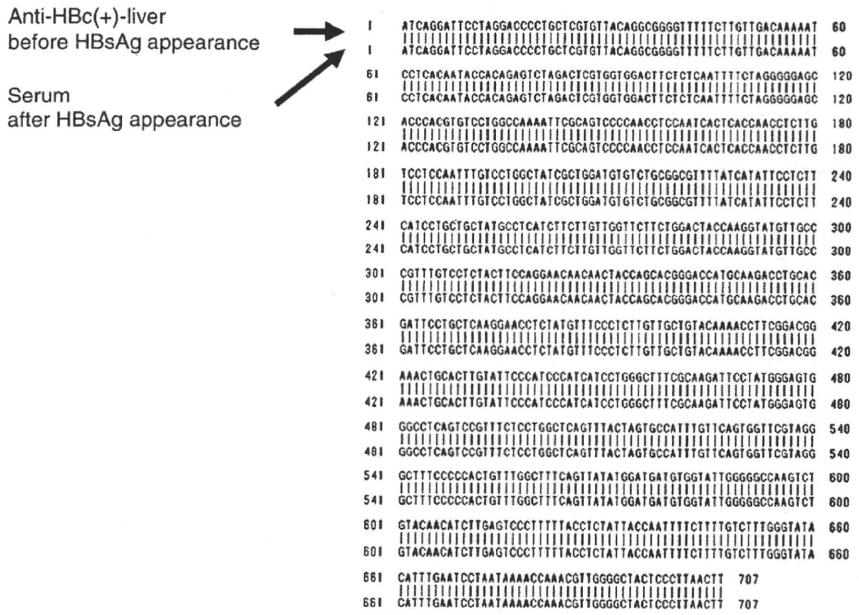

Figure 2 Sequence analyses of hepatitis B virus (HBV) derived from the liver tissue and serum of the hepatocellular carcinoma (HCC) patient who was initially given a diagnosis of chronic hepatitis C virus. The sequence analyses revealed that the nucleotide sequences of HBV surface region derived from the liver tissue at the time of operation for HCC showed 100% homology to that from the serum at the time of hepatitis B surface antigen appearance.

after HBV exacerbation with those detected in the tumor and the surrounding liver tissues at the time of HCC resection. No amplification was obtained by nested PCR using primer set for detection of surface region in the serum sample at the time of operation, HBV DNA was amplified from DNA samples extracted from both the liver tissues obtained at the time of HCC resection and the serum at the time of HBsAg appearance. The detection limit of nested-PCR used in this study was determined as 1×10^2 copies/mL of the circulating viruses.[3] The sequence analyses revealed that the nucleotide sequences of viral surface region derived from the serum showed 100% homology to that from the liver tissues of both cancerous and noncancerous regions (Figure 2), indicating that he had been infected latently with HBV in his liver when he developed HCC, and that HBV reactivation occurred through the clinical course of management of the malignancies.

DISCUSSION

HCC IS A major public health problem worldwide. More than 75% of HCCs are caused by HCV-related chronic liver disease, and nearly 15% are related to HBV-related liver disease.[12] Many reports have suggested the possibility that the *de novo* HBV infection may contribute to the development of HCC in HCV-positive patients.[6–8,13,14] However, the role of occult HBV infection in HCV-related HCC is controversial because of the lack of direct evidence showing infection with HBV in the liver of HCV-positive patients. We provided here for the first time the direct evidence of *de novo* HBV infection in the liver tissue of the HCC patient who was initially given a diagnosis of HCV-related chronic liver disease. The patient described here had an HCV-related chronic liver disease with the serological markers positive for anti-HBc but negative for HBsAg, and developed HCC after the complete eradication of HCV infection by IFN therapy. Although he initially lacked any of the serological markers for HBV antigen or circulating HBV DNA in his sera, he developed the appearance of HBsAg and HBV DNA during the course of the management of a series of cancers.

Except the setting of liver transplantation, *de novo* HBV reactivation has been reported in patients after hematopoietic stem cell transplantation and cytotoxic chemotherapy treatment. We previously reported two cases with fatal fuluminant hepatitis caused by *de novo* HBV reactivation.[15,16] Both cases had initially had anti-HBc but not HBsAg and immunosuppressive condition introduced before bone marrow transplantation or chemotherapy for chronic B-cell leukemia resurlted in the reactivation of *de novo* HBV. Recently, Hui *et al.* showed that intensive chemotherapy triggered the development of *de novo* HBV-related hepatitis in eight of 244 lymphoma patients.[17] These findings indicate that the immunosuppressive state caused by malignancies and chemotherapy may result in enhanced repli-

cation of latently infected HBV in the liver tissues, leading to the appearance of HBsAg and HBV DNA in the sera. Although these clinical findings strongly supported the *de novo* HBV reactivation in anti-HBc-positive individuals lacking initial HBsAg, it remained an unsettled question whether the viruses appeared in the sera of patients under immunosuppressive setting was actually derived from their own liver tissues, not from the exogenous origins such as blood transfusion or drug injection during hospitalization. In the current report, we clearly demonstrated that the sequences of the circulating HBV genome after viral reactivation was completely identical to those of the latently infected HBV DNA detected in the liver tissues of the anti-HBc patient, indicating that latent HBV infection was present in anti-HBc-positive patient with HCV-related chronic liver disease and gave rise the *de novo* HBV reactivation during the clinical course of multiple cancer development. The reason why latently infected HBV was spontaneously reactivated in the current case remains unclear at present, since he never received any intensive chemotherapy against cancers. One possibility is, however, to assume that a series of cancer development might cause the change of immunological status, leading to the viral reactivation. Indeed, it is firmLy established that the immunological capacity of cancer patients can be depressed by the disease itself, and thus cancer patients have impaired immune response and susceptible to any viral infection and/or reactivation.[18]

In conclusion, our case indicates that HCV-positive individuals who are positive for anti-HBc in the absence of HBsAg could have latent HBV infection in their liver tissues. One thing to be noted is that this patient developed HCC after the complete clearance of initially infected HCV by IFN treatment. Interestingly, our recent etiological study revealed that none of the anti-HBc-negative patients with HCV-related chronic hepatitis who had a virological response to IFN therapy developed HCC, whereas HCC was diagnosed in some of the anti-HBc-positive patients with a virological response to anti-HCV therapy.[11] Thus, it is tempting to assume that intrahepatic HBV infection played a pivotal role in the development of HCC after the eradication of HCV in our patient. Indeed, a great deal of solid evidence indicates that occult HBV infection is a risk factor for HCC development.[19-21] Further investigation is needed to determine the mechanisms how occult HBV infection could contribute to hepatocarcinogenesis in patients with positive for anti-HBc.

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SUPPORTING INFORMATION

ADDITIONAL SUPPORTING INFORMATION may be found in the online version of this article:

Table S1 Laboratory data before receiving the interferon therapy

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EDUCATION AND IMAGING

Hepatobiliary and pancreatic: Delayed ileal perforation following radiofrequency ablation of hepatocellular carcinoma

An 80-year-old man was diagnosed with a recurrence of hepatocellular carcinoma on a contrast-enhanced computed tomography (CT) scan (arrow, Figure 1a). The tumor was approximately 8 mm from the liver edge and did not appear to be adjacent to the gastrointestinal tract. After infusion chemotherapy via the hepatic artery, ultrasound-guided radiofrequency ablation was performed under local anesthesia using a single internally cooled electrode with a 2-cm tip exposure. A CT scan obtained 1 day after radiofrequency ablation showed appropriate necrosis of the tumor without any apparent complications (arrow, Figure 1b). However, 14 days after radiofrequency ablation, the patient returned to the Emergency Department with abdominal pain. A repeat CT scan showed free air in the mesentery and thickening of the small bowel wall in the mid-abdomen. An early laparotomy was performed and revealed thermal damage to the ileum with a pinhole-sized perforation (arrow, Figure 2) but the damaged ileum was not adherent to the liver. The damaged segment was resected with an end-to-end anastomosis and the patient had an uneventful recovery.

Radiofrequency ablation is an effective treatment for hepatocellular carcinoma with complication rates that range from 2% to 10%. Early complications include bleeding into the peritoneal or pleural cavities, perforation of the gastrointestinal tract and the development of a liver abscess. Late complications can include seeding of tumor along the electrode track and the development of strictures within the biliary system. In relation to intestinal perforation, a large multicenter study recorded 7 cases in 2320 patients, a frequency of 0.3%. Two of these patients died. The site of the perforation was the transverse colon in 5 patients and this appeared to be associated with adhesions in the upper abdomen. Perforations were also reported in the stomach (1 case) and jejunum (1 case). All patients with perforation had tumor within 1 cm of the liver capsule. In the above patient, the neoplasm was close to the capsule but adjacent bowel was not shown on a CT scan. However, the apparent absence of adjacent bowel does not exclude the possibility of intestinal perforation. Furthermore, the clinical features of perforation can be delayed for at least 2 weeks after radiofrequency ablation.

Contributed by
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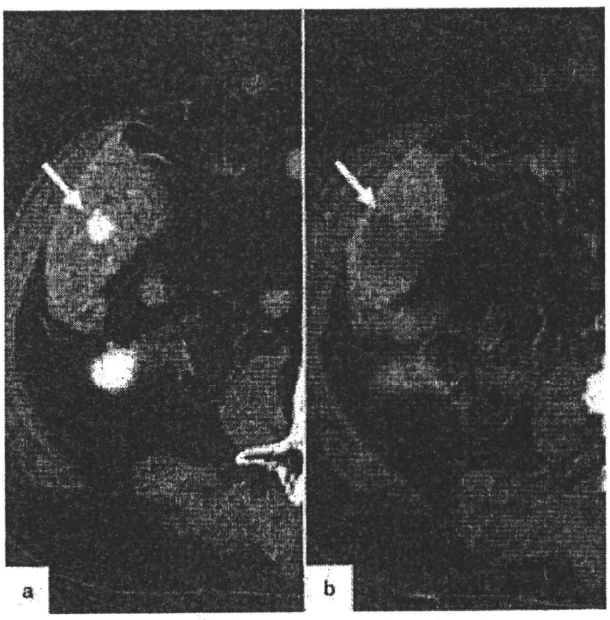

Figure 1

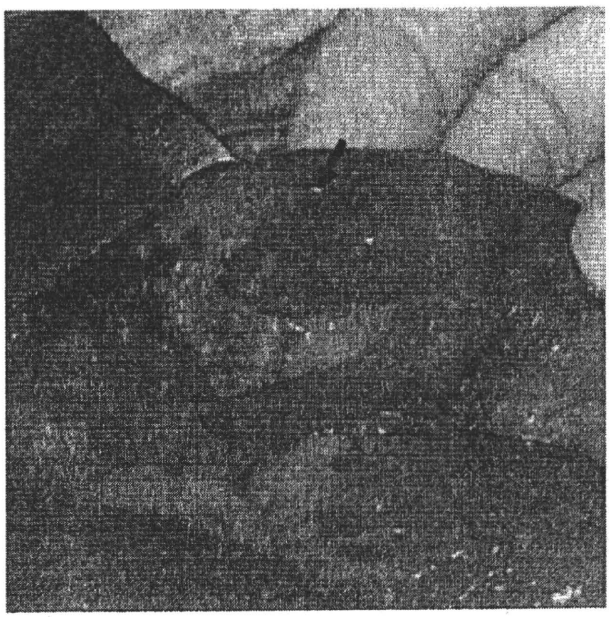

Figure 2

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Laparoscopic findings of reddish markings predict hepatocellular carcinoma in patients with hepatitis B virus-related liver disease

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Abstract
Background For patients with chronic hepatitis due to hepatitis B virus (HBV), factors predicting hepatocellular carcinoma (HCC) other than high levels of HBV-DNA and alanine aminotransferase (ALT) are needed to prevent HCC development, as many patients with chronic HBV infection fulfill these conditions. The purpose of this study was to clarify factors predictive of HCC development for those patients.
Methods The study was a systematic cohort analysis of 303 consecutive patients with hepatitis B e-antigen, receiving laparoscopic examination for assessment of liver disease. Laparoscopic, histological, and clinical characteristics were investigated as related to HCC development.
Results HCC occurred in 27 patients during a mean follow-up of 8.0 ± 5.0 years, at the age of 37–72 years. Significant associations with HCC development were shown for liver cirrhosis, histological activity grade, reddish markings, and older age. Multivariate analysis revealed that HCC development was strongly associated with older age and male gender ($P = 0.002$ and $P = 0.043$, respectively). HCC occurred more frequently in patients of age ≥30 years even with early stage than in patients of age <30 years ($P = 0.031$). Severe reddish markings, a laparoscopic finding of widespread parenchymal destruction, were highly associated with HCC development in patients of age ≥30 years at diagnosis (odds ratio = 1.67, $P = 0.034$), while histological activity grade and ALT level were not ($P = 0.075$ and $P = 0.69$, respectively).
Conclusions HCC development is associated with older age, male gender, and liver cirrhosis. Reddish markings, rather than histological activity or ALT level, can be useful to predict HCC for HBV patients of age ≥30 years.

Keywords Hepatitis B virus · Hepatocellular carcinoma · Laparoscopy

Abbreviations
HBV Hepatitis B virus
HCC Hepatocellular carcinoma
ALT Alanine aminotransferase
HCV Hepatitis C virus
AST Aspartate aminotransferase

Introduction

Hepatitis B virus (HBV) is distributed worldwide, and 400 million people suffer from chronic hepatitis B infection [1]. Hepatocellular carcinoma (HCC) and liver failure are frequent among patients with HBV infection. The incidence

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of HCC development is estimated at 0.8% annually, approximately 100-fold higher than the rate among uninfected people. Half a million patients die of liver-related causes every year [2]. Several studies of the prognosis of HBV have shown that persistent elevation of HBV-DNA and alanine aminotransferase (ALT) in serum are highly associated with rapid disease progression and HCC development [3, 4]. Host factors such as age, gender, and alcohol intake, and viral factors including hepatitis B e-antigen (HBeAg) and HBV genotype have been implicated as important contributors to disease progression. In Japan, HBV genotype C is predominant over other genotypes, and most HBV patients with chronic hepatitis have been infected perinatally or during early childhood [5]. Recent reports have indicated that HBV genotype C is related to poor outcome of slower HBeAg seroconversion [6], earlier disease progression, and more frequent HCC development [7].

Good control of viral replication with nucleoside analogues can decrease liver inflammation and reduce the risk of poor outcomes [8]. Such drugs may work, in the short term at least, for most patients in the immune-active phase of chronic HBV infection. However, benefits for long-term survival have not been well defined. Some patients in young or middle age hesitate to use these drugs due to the possibility of drug resistance and the high cost for medication for life-long use. The presence of HBeAg often indicates active viral replication, and high levels of ALT in the immune-active phase; many patients with HBeAg are thus suitable candidates for use of nucleoside analogues. Predictors for rapid progression to liver cirrhosis and high risk of HCC development should be more clearly defined, to facilitate the selection of HBeAg-positive patients who should be treated immediately with nucleoside analogues.

Laparoscopy provides wide and precise observation of the liver surface. Kalk [9, 10] reported morphological progression from acute hepatitis to cirrhosis. Laparoscopic observation with liver biopsy is considered the most accurate method of evaluating liver cirrhosis [11–14]. Besides usefulness in evaluating present disease progression, direct observation of the liver surface can provide a large amount of information on disease activity, capsular structural changes, and small lesions on the surface, which can be difficult or impossible to detect on ultrasonography (US) or computed tomography (CT). Studies of patients with hepatitis C virus (HCV) have proposed the importance of laparoscopic examination and have noted that irregular regenerative nodules, degree of regenerative nodules, and atrophic right lobe can be observed clearly by laparoscopy, and also that those findings represent independent risk factors for HCC development [15, 16]. Associations with laparoscopic features have not been well defined for HBV patients with regard to HCC development.

The purpose of this study was to clarify useful predictive factors of HCC development for HBV patients with HBeAg, by evaluating laparoscopic features, clinical characteristics, and histology with regard to the development of HCC. We reveal that liver cirrhosis, older age, male gender, and a laparoscopic feature of reddish markings were strongly associated with HCC development, and propose the importance of laparoscopic examination to evaluate the risk of HCC development.

Patients and methods

Patients

This study was a systematic cohort analysis of 303 consecutive patients with HBeAg, and who underwent laparoscopic examination and liver biopsy for the assessment of chronic liver injury at Okayama University Hospital between 1982 and 2002. Presence of HCC was excluded in all patients by imaging examinations with abdominal ultrasonography and computed tomography and by showing normal values of alpha-fetoprotein in serum at the time of diagnosis. Patients suffering from acute hepatitis due to HBV, those with serum positivity for anti-HCV antibodies, and those with daily ethanol intake >75 g were excluded from the study. The study was performed in accordance with the Helsinki Declaration, and all protocols were approved by the ethics committees of the involved institutes. All patients provided informed consent before enrolment into the study.

Scoring of liver function by using laboratory parameters

In order to estimate the usefulness of laboratory parameters to assess liver function, we selected five conventional parameters, and evaluated the score based on these values with histological fibrosis stage. These parameters were scored according to the normal ranges in our institutes as follows: prothrombin time (0, >80%; 1, ≤80%); platelet count (0, $>15 \times 10^4/mm^3$; 1, $\leq 15 \times 10^4/mm^3$); serum level of albumin (0, >3.9 g/dl; 1, ≤3.9 g/dl); serum level of total bilirubin (0, <1.2 mg/dl; 1, ≥1.2 mg/dl); and the ratios of aspartate aminotransferase (AST) and ALT (0, <1.0; 1, ≥1.0).

Histological evaluation

Stage of histological fibrosis and grade of activity were assigned by two pathologists according to the criteria of Desmet et al. [17]. All biopsy specimens were obtained under laparoscopic guidance and were more than 1.5 cm

long and 2 mm wide. The amount of obtained material was therefore adequate for histological evaluation.

Laparoscopic examination

We selected the following six features for analysis, because these are routinely used for evaluation of disease progression and activity: surface irregularity, whitish markings, vascular proliferation, reddish markings, patchy markings, and fat deposition [18–21]. Surface irregularity was evaluated, based on depression and nodular formation, and classified into three stages: S1, normal or early stage; S2, advanced, pre-cirrhotic stage; and S3, cirrhotic stage. Reddish markings were scored according to location, distribution, and color tone of the markings. Whitish markings were defined with their location. These features were assessed as mild or severe based on the total scores as in Table 1. As for vascular proliferation, dilated peripheral portal veins are often observed on liver surface of the patients with chronic hepatitis, and small arteries may become visible when the disease has progressed. We graded dilated peripheral portal veins as mild and proliferation of small arteries as severe for vascular proliferation. These classifications have been used since Shimada et al. [18] reported their usefulness in 1971 to evaluate disease activity and to predict disease progression for chronic hepatitis. Several reports from different institutes have proposed similar classifications by using these features, and revealed their importance for evaluation of disease progression [16, 22, 23]. Final laparoscopic findings were evaluated independently by three experienced hepatologists (S.F., B.S., and K.Y.), and discussed for final diagnosis. Figure 1 shows typical laparoscopic features of the liver surface.

Table 1 Laparoscopic evaluations of reddish markings and whitish markings

Item	Definition	Score
Reddish markings		
Location	Periportal	1
	Pericentral	1
	Multilobular	2
Distribution	Localized	1
	Sparse	2
	Dense	3
Tone of color	Indistinct	1
	Common	2
	Hemorrhagic	3
Diagnostic classification		
None		0 points
Mild reddish marking		<5 points
Severe reddish marking		≥5 points
Whitish markings		
Location	Spotted	1
	Asteroidal	2
	Network-like	2
Diagnostic classification		
None		0 points
Mild whitish marking		<2 points
Severe whitish marking		≥2 points

Follow-up

All patients received medical check-ups with blood examinations every 2–3 months, and abdominal US or CT every 6 months at least as recommended [24, 25]. Patients who had not visited our hospital in the previous 6 months were contacted by letter or telephone and asked to provide details of recent medications by questionnaires. If they visited other hospitals, we also asked them about the results of any imaging studies. For cases in which the patient had died, the date and cause of death were recorded. No patients were treated with nucleoside analogues during follow-up.

Statistical analysis

Data are expressed as mean ± standard deviation (SD) or median (range). Patient laboratory data and laparoscopic findings were compared with histological findings using the Kruskal–Wallis test and canonical correlation analysis. Proportional hazards models were utilized to estimate the effects of patient characteristics on HCC development. Incidence rates of HCC were estimated by using the Kaplan–Meier method, and compared with the log-rank test. A value of $P < 0.05$ was considered significant. Statistical analysis was performed with JMP software (SAS Institute, Cary, NC).

Results

Patient characteristics

Table 2 lists the clinical characteristics of patients enrolled in this study. Mean age of patients was 34 ± 11 years, and 232 patients were male (76.6%). Of the patients, 71.6% had some family history of liver disease. In order to estimate the usefulness of laboratory parameters to assess liver function, we selected five conventional parameters, and compared the scores based on these values with histological fibrosis stage (Fig. 2a). Surprisingly, only half of the patients with a total score of 0 (49.1%), representing completely normal in this scoring system, were histologically defined as early stage

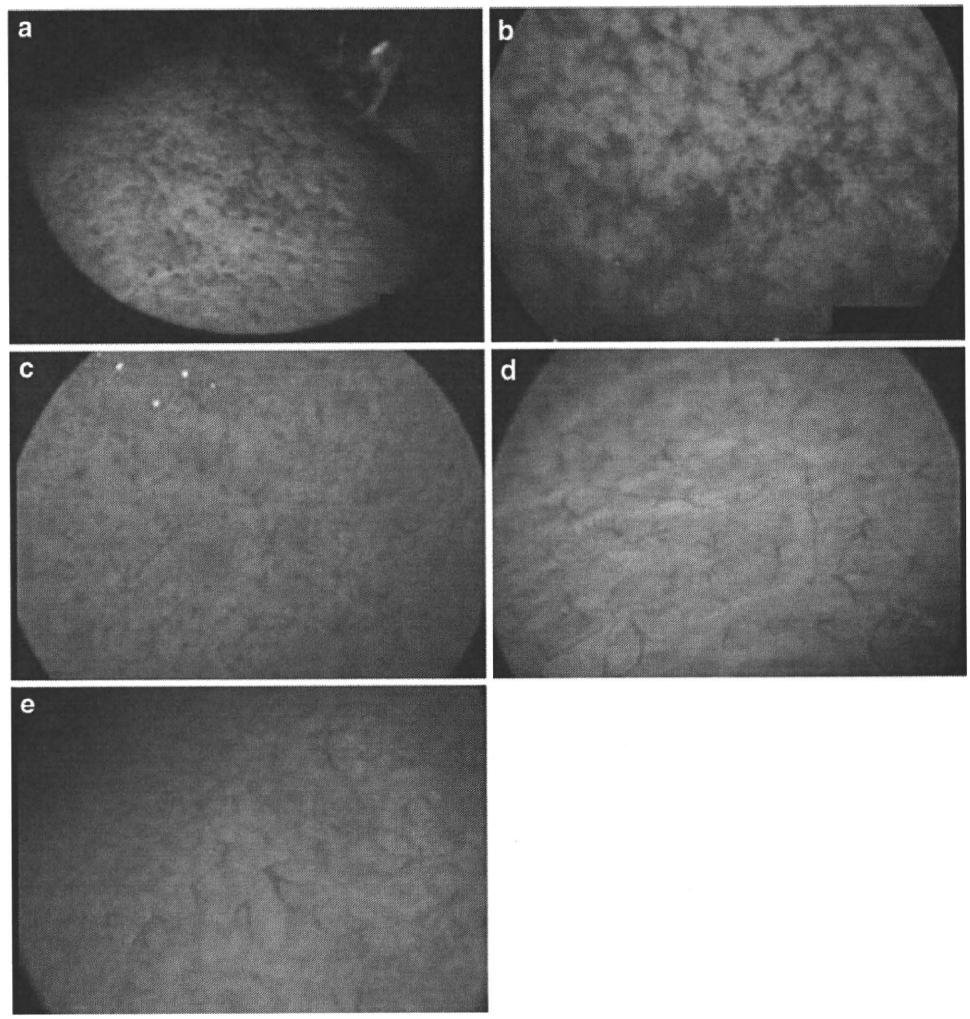

Fig. 1 Laparoscopic features of the patients with chronic viral hepatitis. Figures show typical pictures of laparoscopic features; laparoscopy of severe reddish markings, showing advanced surface irregularity with densely distributed reddish markings (**a**), closer view of severe reddish markings in hemorrhagic color which are multilobularly located (**b**), closer view of mild reddish markings, showing common redness in periportal areas (**c**), laparoscopy of vascular proliferation (**d**), and laparoscopy of normal liver (**e**)

Table 2 Patient characteristics at the time of diagnosis ($N = 303$)

Age at diagnosis (years)	34 ± 11[b]
Gender (female/male)	71/232
Family history of liver disease	217 (71.6%)
History of blood transfusion	14 (4.6%)
Liver histology	
Fibrosis stage (1/2/3/4)[a]	92/90/101/20
Activity grade (1/2/3)[a]	104/135/64
Laboratory data at diagnosis	
AST (IU/l)	91 ± 73[b]
ALT (IU/l)	156 ± 142[b]
Total bilirubin (mg/dl)	0.87 ± 0.53[b]
Albumin (g/dl)	4.2 ± 0.4[b]
Platelet count ($\times 10^4/mm^3$)	18 ± 6[b]

AST aspartate aminotransferase, *ALT* alanine aminotransferase

[a] Histological stage classified according to Desmet et al. [17]

[b] Mean ± SD

(fibrosis stage 0 or 1), and 20.5% were advanced, at the pre-cirrhotic or cirrhotic stage (fibrosis stage 3 or 4). These results indicate the necessity for liver biopsy, as conventional laboratory parameters cannot distinguish patients in the early stage from those in the advanced stages, although total scores of laboratory data correlated significantly with stages of histological fibrosis ($R = 0.46$, $P < 0.0001$, canonical correlation analysis). In terms of activity grades, mean ALT levels in patients were very high (156 ± 142 IU/l), and 51.9% of patients with histological grade A1 showed ALT levels ≥80 IU/l (Fig. 2b). ALT levels displayed weak associations with histological activity grade ($R = 0.14$, $P = 0.013$).

Laparoscopic findings at the time of diagnosis

Table 3 provides a summary of laparoscopic features. Frequencies were calculated for each group of surface

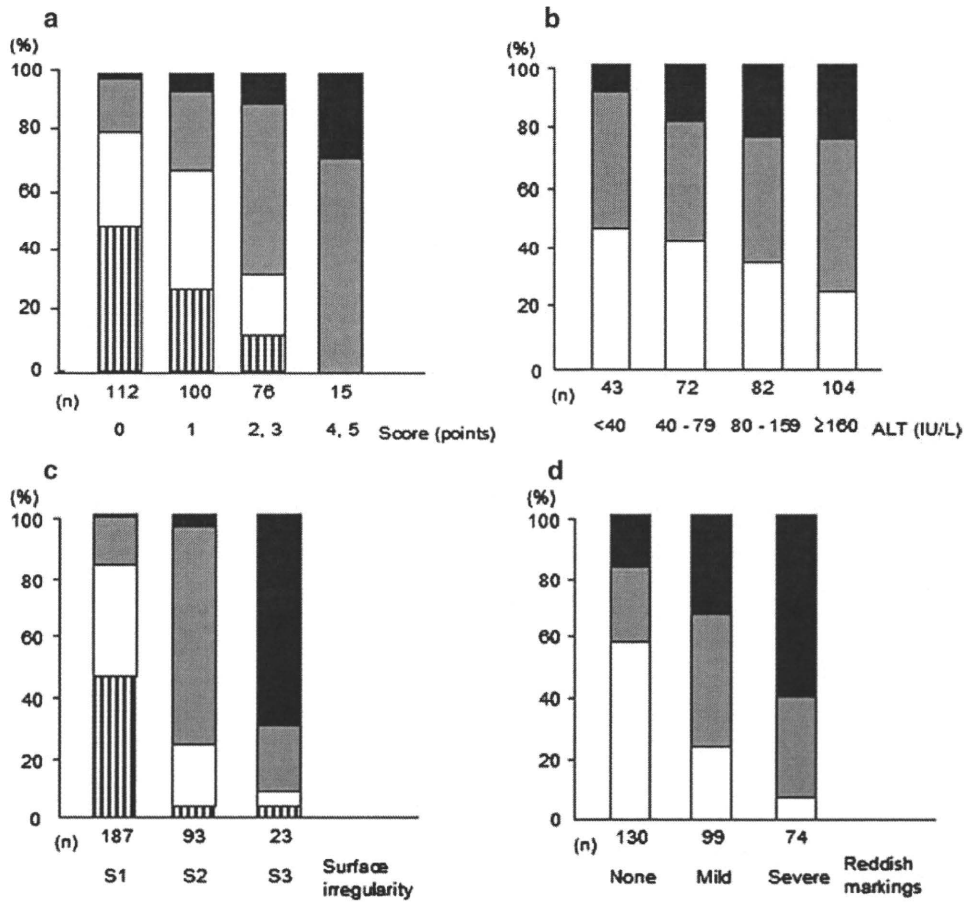

Fig. 2 Comparisons of histology, laboratory parameters, and laparoscopic findings. Histological fibrosis stage was compared with total scores of the five conventional parameters related to liver function with significant correlations ($R = 0.46$, $P < 0.0001$, canonical correlation analysis, **a**): fibrosis stage 1, *striped*; stage 2, *open*; stage 3, *gray*; and stage 4, *black*. Significantly high correlations were also shown between histological fibrosis stage and laparoscopic surface irregularity ($R = 0.66$, $P < 0.0001$, **c**): fibrosis stage 1, *striped*; stage 2, *open*; stage 3, *gray*; and stage 4, *black*. As for the activity, alanine aminotransferase (ALT) levels were divided into four groups and compared with histological activity grade, showing significant associations ($R = 0.14$, $P = 0.013$, **b**): A1, *open*; A2, *gray*; A3, *black*. Correlations between histological activity grade and reddish markings were significant as shown in **d** ($R = 0.45$, $P < 0.0001$): A1, *open*; A2, *gray*; and A3, *black*

Table 3 Summary of laparoscopic features of HBV patients

	Surface irregularity[a]		
	S1 ($n = 187$)	S2 ($n = 93$)	S3 ($n = 23$)
Reddish markings	89 (48%)	69 (74%)	15 (65%)
Severe reddish markings	34 (18%)	34 (37%)	6 (26%)
Whitish markings	51 (27%)	22 (24%)	3 (13%)
Severe whitish markings	34 (18%)	12 (13%)	2 (9%)
Vascular proliferation	160 (86%)	68 (73%)	20 (87%)
Severe vascular proliferation	110 (59%)	57 (61%)	15 (65%)
Patchy markings	28 (15%)	67 (72%)	2 (9%)
Fat deposition	46 (25%)	25 (27%)	11 (48%)

[a] Surface irregularity, classified in three stages: S1, normal or early stage; S2, advanced, pre-cirrhotic stage; and S3, cirrhotic stage

irregularity. Reddish markings and patchy markings were frequently observed in S2 (74 and 72%, respectively, $P < 0.001$ each). Vascular proliferation was observed less in S2 (73%) than in S1 (86%) or S3 (87%, $P = 0.018$, Kruskal–Wallis tests). Severe vascular proliferation, reflecting proliferation of small arteries, was more frequently observed in S3 than S1 or S2, although this increase was not statistically significant ($P = 0.84$). Whitish markings tended to be less frequent, and fat deposition more frequent in S3 than in S1 or S2, but no significant differences were identified ($P = 0.31$ and $P = 0.061$, respectively). Correlations between histological fibrosis stage and laparoscopic surface irregularity were significantly strong ($R = 0.71$, $P < 0.0001$, canonical correlation analysis; Fig. 2c). Reddish markings were significantly associated with histological activity grade as shown in Fig. 2d ($R = 0.45$, $P < 0.0001$).

Risks of HCC development

HCC development was evaluated for 250 patients who were observed for ≥1 year. The accumulated observation was 1991 person-years, accounting for 80% of the total potential follow-up. HCC developed in 27 patients during a mean follow-up period of 8.0 ± 5.0 years, at the age of 37–72 years. The incidence of HCC development was estimated as 5.7% at 5 years of follow-up, 13.5% at 10 years, and 20.6% at 15 years (Fig. 3a). Figure 3b shows cumulative rates of HCC development by age, estimated as 1.3% at 40 years old, 12.3% at 50 years old, and 27.2% at

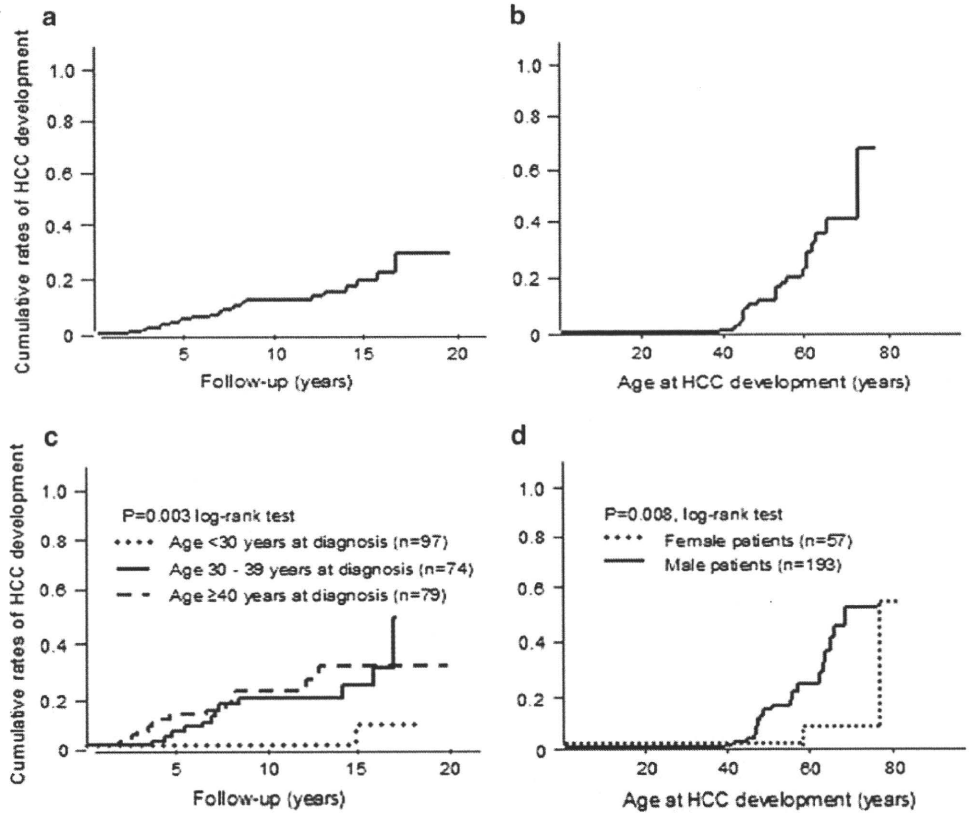

Fig. 3 Cumulative rates of hepatocellular carcinoma (HCC) development. **a** Shows cumulative rate of HCC development as a function of the follow-up period, estimated by the Kaplan–Meier method. The incidence of HCC development was estimated as 5.7% at 5 years of follow-up, 13.5% at 10 years, and 20.6% at 15 years. **b** Shows cumulative rates of HCC development by age, estimated as 1.3% at 40 years old, 12.3% at 50 years old, and 27.2% at 60 years old. When the patients were divided into three groups according to age at diagnosis (<30, 30–39, ≥40 years), there were significant differences in cumulative rates of HCC development among the groups ($P = 0.003$, log-rank test, **c**). Furthermore, **d** shows significant difference in cumulative rates of HCC development between the female patients and the male patients ($P = 0.008$, log-rank test)

60 years old. When the patients were divided into three groups according to age at diagnosis (<30, 30–39, ≥40 years), there were significant differences in cumulative rates of HCC development among the groups ($P = 0.003$, log-rank test; Fig. 3c), especially between the age groups <30, and ≥30 years ($P = 0.0009$, log-rank test). The patient groups of age 30–39 years and age ≥40 years were estimated to have similar risks of HCC occurrence ($P = 0.57$, log-rank test). Furthermore, male patients showed a higher risk of HCC development than females ($P = 0.008$, log-rank test; Fig. 3d), as previously reported [1–7]. Table 4 shows evaluations of clinical characteristics, histology, and laparoscopic features, with regard to HCC development using proportional hazards models. Significant associations with HCC development were shown for liver cirrhosis according to histological fibrosis and laparoscopic surface irregularity, high histological activity grade, laparoscopic severe reddish markings, and older age at diagnosis in univariate analysis. Cumulative risks of HCC development were also estimated by the Kaplan–Meier method (Fig. 4). Severity of reddish markings correlated significantly with risk of HCC development ($P = 0.036$, log-rank test), while histological activity grade did not ($P = 0.054$), suggesting some difference between these two parameters. Multivariate analysis, adjusted with a logistic likelihood ratio test, revealed that HCC development was strongly associated with older age and male gender ($P = 0.002$ and $P = 0.043$, respectively). Laparoscopic surface irregularity was not used for multivariate analysis, due to high correlations of laparoscopic surface irregularity with histological fibrosis stage as shown in Fig. 2c.

Subgroup analysis for HCC development

Next, we studied age difference by dividing patients according to age at diagnosis (<30, and ≥30 years), and our results in proportional hazards models showed that advanced stages according to histological fibrosis stage and surface irregularity were significantly associated with HCC development for patients of age ≥30 years at diagnosis ($P = 0.040$ and $P = 0.016$, respectively; Table 5). Severe

Table 4 Analysis of factors predicting HCC development with the proportional hazards model

Factors	Univariate analysis		Multivariate analysis	
	Odds ratio (range[a])	*P*	Odds ratio (range[a])	*P*
Age at diagnosis (years)	1.06 (1.03–1.10)	<0.001	1.06 (1.02–1.11)	0.002
Gender (male)	3.32 (0.78–14.0)	0.10	4.53 (1.05–19.6)	0.043
Blood transfusion	2.40 (0.56–10.2)	0.24		
Family history of liver disease	1.46 (0.67–3.18)	0.35		
Interferon therapy	0.65 (0.29–1.45)	0.29		
Histological fibrosis stage	1.80 (1.18–2.76)	<0.001	1.21 (0.71–2.07)	0.49
Histological activity grade	1.82 (1.06–3.14)	0.031	1.16 (0.58–2.34)	0.68
AST (≥80 IU/l)	1.32 (0.62–2.83)	0.47		
ALT (≥80 IU/l)	1.06 (0.48–2.37)	0.88		
Surface irregularity	2.45 (1.46–4.09)	<0.001		
Whitish markings	0.77 (0.31–1.90)	0.57		
Vascular proliferation	1.27 (0.48–3.36)	0.64		
Reddish markings	1.66 (1.04–2.65)	0.036	1.45 (0.54–3.90)	0.46
Patchy markings	2.04 (0.96–4.36)	0.065	1.38 (0.57–3.32)	0.48
Fat deposition	1.28 (0.48–3.37)	0.62		

AST aspartate aminotransferase, *ALT* alanine aminotransferase

[a] 95% confidence interval

inflammatory activity with reddish markings also affected HCC development ($P = 0.034$). Therefore we estimated cumulative rates of HCC development, by using the Kaplan–Meier method. Among patients of age ≥30 years at diagnosis, cumulative rates of HCC development were higher in more advanced disease, according to surface irregularity (Fig. 5b, $P = 0.043$, log-rank test). Cumulative rates of HCC development were 37.1% at the 10-year follow-up among the patients in cirrhotic S3 stage, 25.6% among those in pre-cirrhotic S2 stage, and 10.1% among those in S1 stage. Interestingly, the risk of HCC occurrence was significantly higher for those in as early as S1 stage, compared with the patients of age <30 years (Fig. 5c, $P = 0.031$, log-rank test). Actually, none of the patients of age <30 years experienced HCC during the 10-year follow-up. Further subgroup analysis in those of age ≥30 years in each laparoscopic stage could not find any significant factors contributing to HCC development. As for the effects of inflammatory activity on HCC development, significant differences in cumulative rates of HCC development were observed among the patients of age ≥30 years at diagnosis when stratified by reddish markings (Fig. 6, $P = 0.025$, log-rank test), but not by histological activity ($P = 0.087$) or ALT levels ($P = 0.69$).

Discussion

Persistent elevation of HBV-DNA and ALT are associated with rapid disease progression and HCC development [3, 4]. Most patients with HBeAg might be candidates for treatment with nucleoside analogues, as the presence of HBeAg often indicates active viral replication and high levels of ALT in an immune-active state of chronic infection. However, due to drug resistance and the high cost of life-long medication, predictors for HCC development should be more clearly defined so that patients can judge the necessity of immediate treatment using nucleoside analogues. We hypothesized that laparoscopic observation of the liver surface might work for this purpose. The present study retrospectively evaluated long-term outcomes for a large systematic cohort of HBeAg-positive patients, focusing on HCC development, using laparoscopic, histological, and clinical characteristics.

In the present study, half of patients with early-stage (S1) disease were <30 years old at diagnosis. Cumulative rate of HCC development was 0.0% during the following 10 years, partly because some patients showed seroconversion to negative HBeAg in the following 10 years with cessation of hepatitis. Conversely, the patients who were ≥30 years old in the early stage showed a significantly higher risk of HCC, compared with the patients of age <30 years. Treatment with nucleoside analogues may be worth considering in such patients, although incidence rates were less than those of patients in the pre-cirrhotic or cirrhotic stage. Age differences in disease progression have been reported with other chronic liver diseases, including chronic hepatitis C [26], autoimmune hepatitis [27], and primary biliary cirrhosis [28]. Our results suggest that age difference plays some role in HCC development among

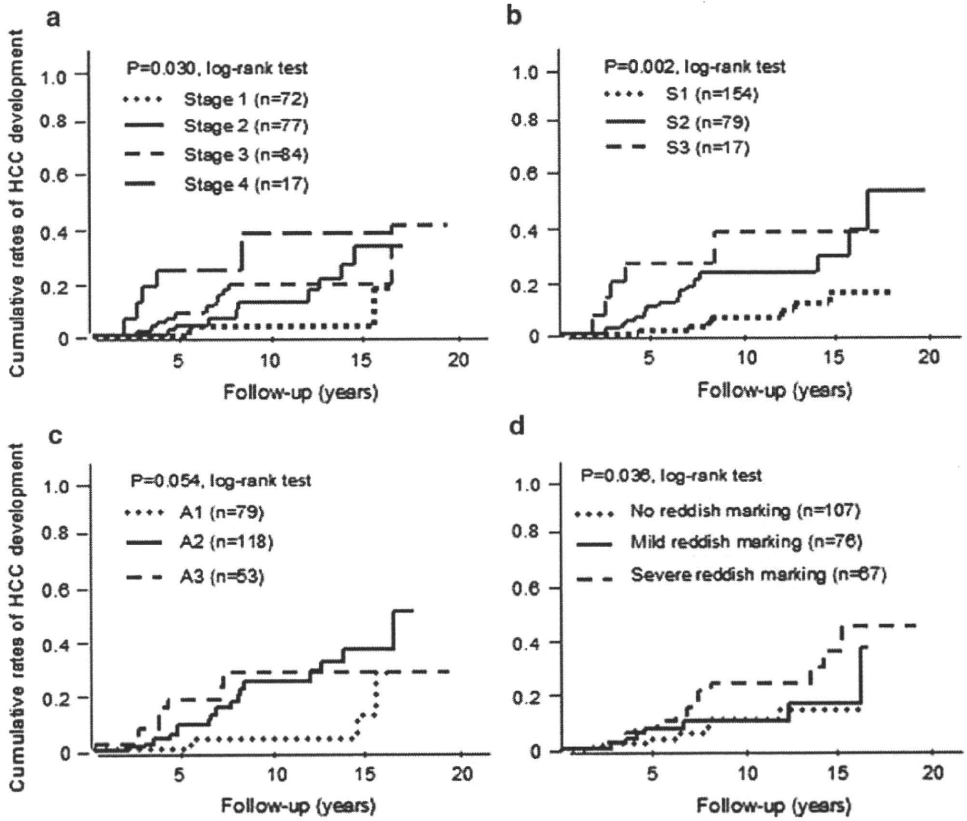

Fig. 4 Cumulative rates of hepatocellular carcinoma (HCC) development, stratified by histology and laparoscopic findings. Figures show cumulative rates of HCC development estimated by the Kaplan–Meier method, stratified by histological fibrosis stage (**a**), laparoscopic surface irregularity (**b**), histological activity grade (**c**), and laparoscopic reddish markings (**d**). Significant associations with HCC development were shown for liver cirrhosis according to histological fibrosis ($P = 0.030$, log-rank test) and laparoscopic surface irregularity ($P = 0.002$). Severity of laparoscopic reddish markings was significantly associated with HCC development ($P = 0.036$), while that of histological activity grade was not ($P = 0.054$, log-rank test)

Table 5 Analysis of factors predicting HCC development for patients of age ≥30 years with the proportional hazards model

Factors	Univariate analysis	
	Odds ratio (range[a])	*P*
Histological fibrosis stage	1.57 (1.02–2.40)	0.04
Histological activity grade	1.67 (0.95–2.95)	0.075
AST (≥80 IU/l)	0.72 (0.33–1.57)	0.41
ALT (≥80 IU/l)	1.18 (0.53–2.66)	0.69
Surface irregularity	1.93 (1.13–3.31)	0.016
Reddish markings	1.67 (1.04–2.70)	0.034
Patchy markings	1.54 (0.68–3.48)	0.30

AST aspartate aminotransferase, *ALT* alanine aminotransferase

[a] 95% confidence interval

HBV patients, and that patients of age <30 years should be re-evaluated with liver biopsy within 10 years if HBV-DNA and ALT levels remain elevated.

Interestingly, our analysis of the patients of age ≥30 years revealed that a laparoscopic finding of reddish markings correlated significantly with HCC development. Reddish markings were significantly correlated with histological activity, but these parameters showed different influences on HCC development. This was suspected to arise from differences in the origins of these parameters. Ohta et al. performed precise histological analysis of reddish markings with histological reconstruction using serial sections of liver biopsy specimens from cases with reddish markings [24]. They revealed that reddish markings correspond to widespread necrosis of hepatocytes, and proposed this finding as a useful index of activity in chronic hepatitis. Shibayama et al. [16, 23] showed that reddish markings did not appear in the early stage of chronic hepatitis with piecemeal necrosis around the portal area, instead appearing only after hepatic parenchymal destruction subjacent to the liver capsule due to prolonged active hepatitis or repeated acute exacerbations of chronic hepatitis. Reddish markings as an index of laparoscopic activity are not equivalent to piecemeal necrosis as an index of histological activity. Progression to liver cirrhosis may occur after the appearance of reddish markings unless the activity of chronic hepatitis can be reduced, because hepatic parenchymal destruction may change the pattern of blood flow in the liver to an increasingly cirrhotic pattern. Reddish markings might be useful not for early detection of HCC, but as a warning of transition to liver cirrhosis prior to HCC development. Our results indicate reddish markings as a useful predictor of HCC development.

In terms of liver cirrhosis, our results are consistent with previous reports, showing that liver cirrhosis in histological fibrosis or laparoscopic surface irregularity is strongly associated with HCC development [14]. This strong association might explain the results of subgroup analysis

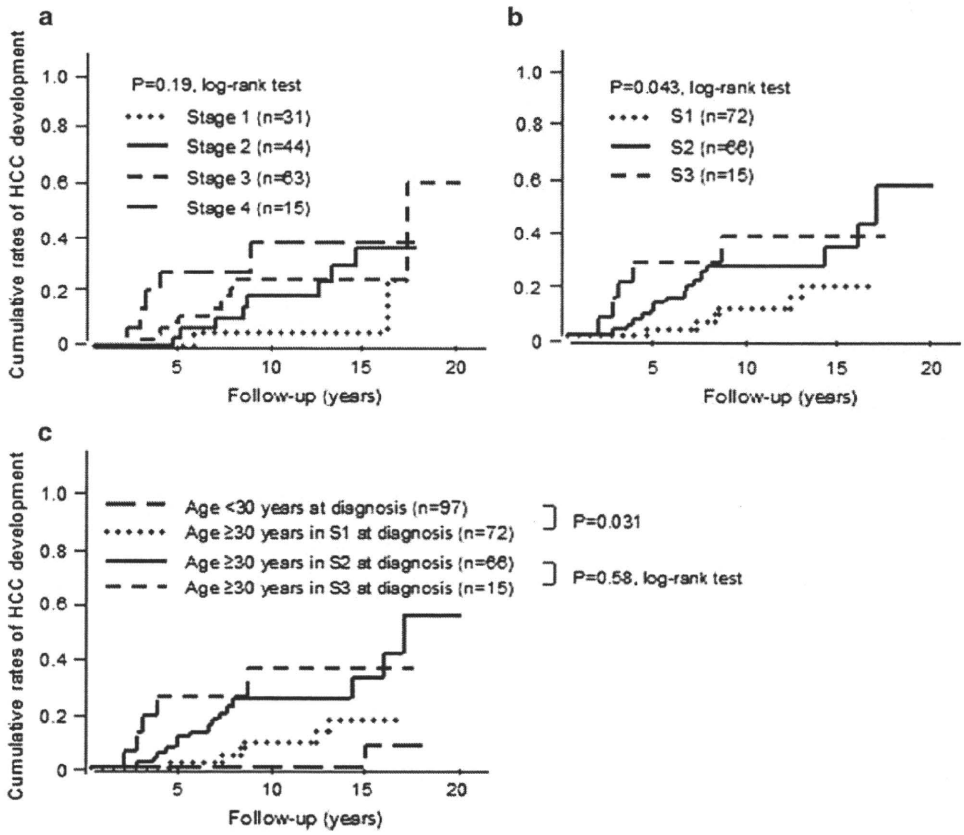

Fig. 5 Cumulative rates of hepatocellular carcinoma (HCC) development, for the patients of age ≥30 years at diagnosis, stratified by disease progression. Figures show cumulative rates of HCC development, for the patients of age ≥30 years at diagnosis, stratified by histological fibrosis stage (**a**) and surface irregularity (**b**, **c**). Cumulative rates of HCC development were significantly higher in more advanced diseases, according to surface irregularity (**b** $P = 0.043$, log-rank test), but not to histological fibrosis stage (**a** $P = 0.19$). The risk of HCC development was significantly higher among the patients of age ≥30 years even in laparoscopic S1 stage at diagnosis, compared with the patients of age <30 years (**c** $P = 0.031$, log-rank test)

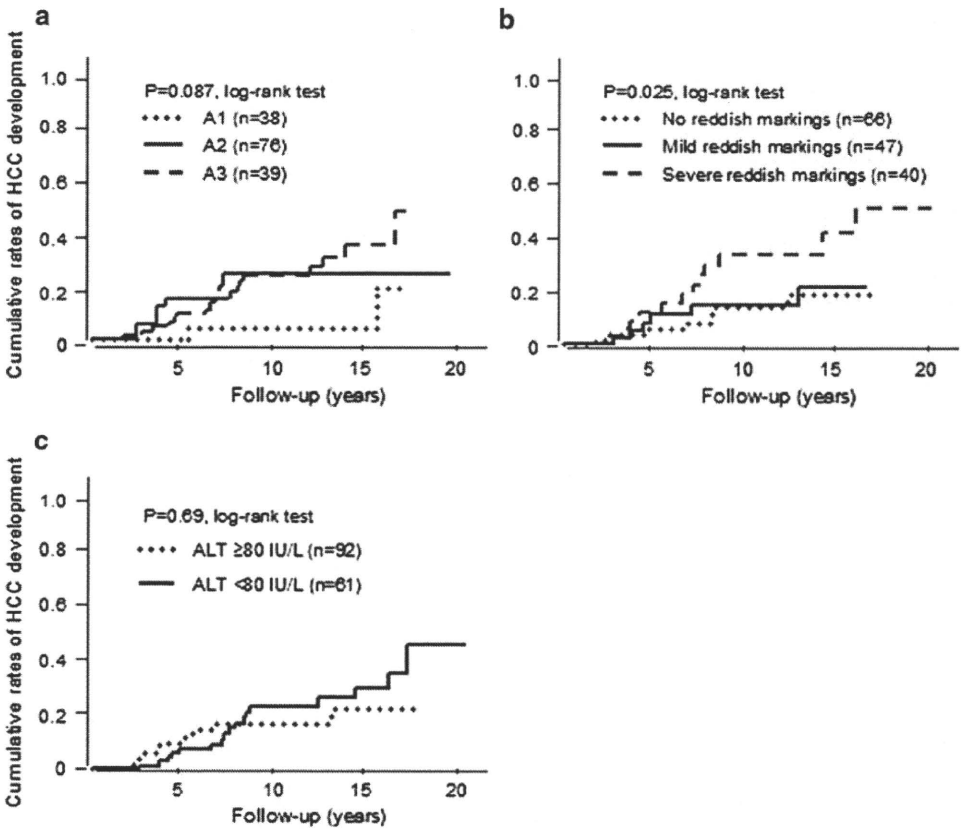

Fig. 6 Cumulative rates of hepatocellular carcinoma (HCC) development, for the patients of age ≥30 years at diagnosis, stratified by inflammatory activity. Cumulative rates of HCC development are shown for patients of age ≥30 years at diagnosis, stratified by histological activity (**a**), laparoscopic reddish markings (**b**), and ALT levels (**c**). Cumulative rates of HCC development showed significant differences when stratified by reddish markings ($P = 0.025$, log-rank test), but not by histological activity ($P = 0.087$) or ALT levels ($P = 0.69$)

among cirrhotic patients, in which no significant predictive factors could be found for HCC development. This reveals that HCC might occur irrespective of other conditions such as liver inflammation, once liver disease has progressed to cirrhosis. Actually, the role of antiviral therapy with nucleoside analogues has not been well defined for cirrhotic patients with regard to reduced HCC development. We have previously reported that cumulative recurrence rates of HCC after initial and complete treatment for HCC did not differ between lamivudine-treated and control groups [29]. Kuzuya et al. [30] supported this finding and suggested that antiviral therapy may improve remnant liver function and increase the chances of receiving available treatment modalities for recurrent HCC.

Completely normal values from routine laboratory tests of liver function might suggest a normal liver or only early-stage liver disease, but our analysis showed that only half of patients with such completely normal values were in the early stage. Several investigators have reported noninvasive approaches for quantitative diagnosis of liver fibrosis, using routine laboratory tests, serum fibrosis markers, radiological imaging, and elastography [31], all of which have been in practical use for hepatitis C. Prolonged active hepatitis or repeated acute exacerbations may occur frequently in HBV patients, and might disturb the accuracy of noninvasive quantitation of liver fibrosis [32]. Liver biopsy appears warranted for precise evaluation of disease progression, and further examination with laparoscopy would be ideal, even if liver function tests continue to yield normal results.

In conclusion, HCC development is associated with older age, male gender, and liver cirrhosis. Reddish markings, rather than histological activity or ALT level, can be useful to predict HCC for HBV patients of age $\geq$30 years at diagnosis. Patients of age $\geq$30 years even in the early stage may consider treatment with nucleoside analogues because of the relatively high risk of HCC development.

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