Impact of Hepatitis B Virus Integration Into Liver Tissue on the Efficacy of Peginterferon and Ribavirin Therapy in Hepatitis B Virus-negative Chronic Hepatitis C Patients

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Background: Integration of hepatitis B virus (HBV) DNA into host hepatic DNA is found in patients without HBV surface antigen (HBsAg). We investigated the prevalence of HBV integration and its association with the outcome of peginterferon (PEG-IFN) and ribavirin combination therapy in HBsAg-negative chronic hepatitis C patients.

Study: We analyzed 157 patients chronically infected with hepatitis C virus (HCV) with viral load $\geq 5.0 \log_{10}$ IU/mL, who underwent PEG-IFN and ribavirin combination therapy. HBV integration was measured by an Alu-PCR assay with liver specimens obtained by needle biopsy before treatment.

Results: HBV integration was identified in 54 of the 157 (34.4%) patients. There were no significant differences between patients with and without HBV integration with regard to baseline characteristics including liver histology, pretreatment HCV RNA levels, and genetic polymorphisms near the *IL28B* gene. In patients with HCV genotype 1b (n = 91), a more favorable viral response was observed in patients with HBV integration during therapy, with higher rates of rapid and complete early virologic response. The rate of sustained virologic response (SVR) was significantly higher in patients with HBV integration than those without ($P = 0.0098$). Multivariate analysis identified HBV integration and *IL28B* polymorphisms as independent factors associated with SVR.

Conclusions: HBV integration was associated with favorable viral responses and a higher SVR rate to combination therapy with PEG-IFN and ribavirin in patients infected with HCV genotype 1b. Further studies will be required to confirm this association and elucidate its underlying mechanisms.

Key Words: chronic hepatitis C, early virologic response, hepatitis B virus integration, peginterferon and ribavirin combination therapy, rapid virologic response, sustained virologic response

(*J Clin Gastroenterol* 2014;48:73–79)

Received for publication November 29, 2012; accepted May 13, 2013.
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Supplemental Digital Content is available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions of this article on the journal's Website, www.jcge.com.

The current standard antiviral therapy for patients with chronic hepatitis C consists of combination therapy with peginterferon (PEG-IFN) and ribavirin.[1] Many investigators have identified baseline factors predicting the treatment outcome of PEG-IFN and ribavirin combination therapy in patients infected with hepatitis C virus (HCV). These include viral factors such as HCV genotype and viral load, amino acid substitutions of the HCV NS5A region (interferon sensitivity-determining region) and amino acid substitution of the HCV core region, and host factors such as hepatic steatosis and genetic polymorphisms near the *IL28B* gene (rs12979860 or rs8099917).

Presence of hepatitis B virus (HBV) may affect the clinical course of chronic hepatitis C[2] and increase the risk of hepatocarcinogenesis[3] even in the absence of circulating HBV surface antigen (HBsAg). Furthermore, several studies have investigated the association between HBV integration into liver tissue DNA and hepatocellular carcinoma (HCC) in HBsAg-negative and serum HBV-DNA–negative patients with chronic HCV infection.[4–6] However, the effect of HBV integration on the efficacy of PEG-IFN and ribavirin antiviral therapy for eradicating HCV has not been evaluated. In this present study, we attempted to clarify whether HBV integration affects the efficacy of PEG-IFN and ribavirin combination therapy in HBsAg-negative patients with chronic HCV infection.

MATERIALS AND METHODS

Patients

A total of 165 HBsAg-negative patients infected with HCV and with pretreatment HCV RNA levels $\geq 5.0 \log_{10}$ IU/mL, based on a quantitative real-time polymerase chain reaction (PCR) based method,[7,8] underwent combination antiviral therapy with PEG-IFN and ribavirin between April 2005 and March 2007. Among these patients, 157 patients underwent ultrasound-guided fine-needle liver biopsy 1 to 2 months before the start of the therapy, and liver tissue for DNA analysis was available. These 157 patients made up the study population. This study did not include any patients with a pretreatment HCV RNA level $< 5.0 \log_{10}$ IU/mL because the use of ribavirin along with PEG-IFN is not allowed by Japanese National Medical Insurance System for patients with pretreatment HCV RNA levels $< 5.0 \log_{10}$ IU/mL. None of the patients had a history of intravenous drug use, tattooing, or acupuncture. All patients were negative for serum HBV DNA and HIV antibodies. None had a history of acute hepatitis B.

The study protocol conformed to the ethics guidelines in the Declaration of Helsinki. All patients provided written

informed consent for analysis of biopsy specimens, and the hospital's Institutional Review Board approved the study.

Antiviral Combination Therapy and the Definition of Viral Response

All patients received weekly PEG-IFN α-2b (Pegintron, MSD, Co., Tokyo, Japan) and daily ribavirin (Rebetol, MSD, Co.). The doses of PEG-IFN α-2b and ribavirin were adjusted based on body weight. Patients weighing ≤45 kg were given 60 μg of PEG-IFN α-2b once a week, those weighing >45 and ≤60 kg were given 80 μg, those weighing >60 and ≤75 kg were given 100 μg, those weighing >75 and ≤90 kg were given 120 μg, and those weighing >90 kg were given 150 μg. Patients weighing ≤60 kg were given 600 mg of ribavirin per day, those weighing >60 and ≤80 kg were given 800 mg per day, and those weighing >80 kg were given 1000 mg per day. Dose modifications of PEG-IFN or ribavirin were based on the manufacturer's recommendations. All patients with HCV genotype 1 were scheduled to undergo 48 weeks of treatment and all patients with HCV genotype 2 were scheduled to undergo 24 weeks of treatment. In some patients with HCV genotype 1 whose serum HCV RNA remained positive for 24 weeks after starting therapy, treatment was discontinued before 48 weeks because they had a low likelihood of achieving a sustained virologic response (SVR).

With regard to the treatment outcome, SVR was defined as undetectable serum HCV RNA at 24 weeks after the end of therapy. A patient was considered to have relapsed when serum HCV RNA levels became detectable between the end of treatment and 24 weeks after completing the treatment, despite undetectable serum HCV RNA levels during and at the end of therapy. A nonresponse was defined as detectable serum HCV RNA 24 weeks after beginning therapy (ie, null response or partial nonresponse according to the American Association for the Study of Liver Diseases guidelines[1]). With regard to viral response during therapy, patients were considered to have a rapid virologic response (RVR) if they had undetectable serum HCV RNA 4 weeks after starting therapy. An early virologic response (EVR) was defined as the disappearance or a decrease in serum HCV RNA levels by at least 2 $\log_{10}$ 12 weeks after starting therapy. Patients were considered to have a complete EVR if the serum HCV RNA levels were undetectable 12 weeks after starting therapy and a partial EVR, if the serum HCV RNA levels were detectable but had decreased by at least 2 $\log_{10}$, 12 weeks after beginning therapy. A non-EVR was defined as a lack of decrease by >2 $\log_{10}$ at 12 weeks as compared with pretreatment levels.

Sample Preparation for the Analysis of HBV Integration into Liver Tissue

DNA was extracted from liver tissues obtained by liver biopsy with a QIAamp DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Genomic DNA was stored at 4°C and carefully handled to avoid contamination with nucleic acids. The amplified viral-host junctions were purified with an Qiaquick Gel extraction kit (Qiagen) and sequenced using a Prism Taq DyeDeoxy Terminator Cycle sequencing kit (Applied Biosystems, Foster Carlsbad, CA), according to the manufacturer's instructions. Products were precipitated with ethanol and analyzed with a 3130xl DNA sequencer (Applied Biosystems).

Detection of Viral-Host Junctions

A PCR-based technique (Alu-PCR) was employed using specific primers to human Alu sequences and to HBV sequences, to efficiently amplify viral-host junctions as described previously.[9] Briefly, HBV DNA was amplified from 100 ng of the extracted DNA in a total volume of 50 mL in the presence of 10 pM of each primer and 2.5 U of recombinant Taq (rTaq) polymerase (Toyobo, Osaka, Japan). PCR was performed in a DNA thermal cycler (Perkin Elmer Cetus, Norwalk, CT) with 35 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 1 minute, an initial denaturation at 94°C for 2 minutes, and a final extension at 72°C for 10 minutes. Primers for Alu repeats were 5'-CAGUGCCAAGUGUUUGCUGACGCCA AAGUGCUGGGAUUA-3' (sense) and 5'-AUUAACCC UCACUAAAGCCUCGAUAGAUYRYRCCAYUGCAC-3' (antisense), and Tag sequence of 5'-CAAGTGTTTGCTG ACGCCAAAG-3' (sense) and 5'-ATTAACCCTCACTAA AGCCTCG-3' (antisense).[10] Sample preparation and mixing were carried out in a different room from the one in which the amplified samples were handled, and a filter pipette was used for all steps. Results were considered as valid only if the same results were obtained in at least 2 separate experiments. The amplified viral-host junctions were determined by direct sequencing.[10,11]

Direct Sequencing

The amplified viral-host junctions were purified with an Easy Trap kit (Takara, Otsu, Japan) and sequenced using a Prism Taq DyeDeoxy Terminator cycle sequencing kit (Applied Biosystems), according to the manufacturer's instructions. Products were precipitated with ethanol and analyzed with a 377 Prism DNA Sequencer (Applied Biosystems). To identify the HBV integration site, we used BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) to compare sequences adjacent to the integrated HBV DNA with the human genome.

Other Serological and Virological Tests for HBV and HCV

HBsAg and HBV core antibody (HBcAb) were measured with ARCHITECT HBsAg QT and anti-HBc (Abbott Japan, Tokyo, Japan). Serum HBV DNA was measured by a quantitative PCR assay (COBAS AmpliPrep/COBAS TaqMan HBV Test version 2.0; Roche Molecular Systems, Pleasanton, CA, lower limit of quantification: 2.1 $\log_{10}$ copies/mL, lower limit of detection: 2.0 $\log_{10}$ copies/mL). HCV genotype was determined by PCR with genotype-specific primers.[12,13] HCV RNA concentration was measured with a quantitative PCR assay (COBAS AmpliPrep/COBAS TaqMan HCV Test; Roche Molecular Systems, lower limit of quantification: 1.6 $\log_{10}$ IU/mL, lower limit of detection: 1.2 $\log_{10}$ IU/mL).[7,8]

Analysis of Genetic Polymorphisms near the IL28B Gene

Genotyping of polymorphisms near the *IL28B* gene (rs8099917) was performed using the TaqMan SNP assay (Applied Biosystems) according to the manufacturer's guidelines. A predesigned and functionally tested probe was used for rs8099917 (C__11710096_10; Applied Biosystems). The genetic polymorphisms of rs8099917 correspond to those of rs12979860 more than 99% in Japanese ethnicity.[14] TT rs8099917 genotype corresponds to CC rs1279860 genotype, GG rs8099917 genotype corresponds to TT

rs1279860 genotype, and TG rs8099917 heterozygote corresponds to CT rs1279860 heterozygote.

Statistical Analyses

Data are expressed as means ± SD. Differences in the proportion of patients with and without HBV integration were analyzed using the χ^2 test. Differences in quantitative values were analyzed using the Mann-Whitney U test. Univariate and multivariate analyses using a logistic regression model were performed to identify factors that predict a SVR, including age, sex, body weight, serum alanine aminotransferase activity, serum aspartate aminotransferase activity, serum γ-glutamyl transpeptidase levels, serum alkaline phosphatase values, serum albumin levels, total serum bilirubin values, white blood cell counts, hemoglobin, platelet counts, hepatitis activity grade (A0 and A1 vs. A2 and A3), liver fibrosis grade (F0 and F1 vs. F2 and F3), pretreatment HCV RNA levels, genetic polymorphism of rs8099917 near the *IL28B* gene (TT vs. TG or GG), and HBV integration into the liver tissue (positive vs. negative). We first performed univariate analyses for each factor if they were associated with SVR. After that, multivariate analyses were performed including only factors that were associated with SVR by univariate analyses. The JMP statistical software package, version 4.0 (SAS Institute, Cary, NC) was used for all statistical analyses. All P values were derived from 2-tailed tests, and $P < 0.05$ was considered to indicate a statistical significance.

RESULTS

Integration of Hepatitis B Viral Genome and Patient Characteristics

The sensitivity of PCR amplification was first determined with cells from the hepatoma cell line Huh-2. When we made a 10-fold serial dilution of Huh-2 cells with normal human PBMC from patients without a history of liver disease, we could detect viral-host junctions at approximately 100 copies per PCR.

The clinical characteristics of the study patients are summarized in Table 1. There were 76 men and 81 women with a mean age of 57.7 ± 10.2 years. The HCV genotype was 1b in 91 patients (58.0%), 2a in 50 patients (31.8%), and 2b in 16 patients (10.2%). No patients were infected with HCV genotype 1a because this genotype is usually not found in the Japanese general population. Thirty-two patients had a history of blood transfusion. None of the patients had a history of intravenous drug use, tattooing, or acupuncture. All patients were negative for serum HBV DNA and HIV antibodies. None had a history of acute hepatitis B or an episode of exposure to HBV. HBcAb was positive with low titer in 26.8% of patients. With regard to treatment outcome, 83 patients (56.1%) achieved SVR, 41 patients (27.7%) experienced relapse, and the remaining 24 patients (16.2%) experienced nonresponse. The rates of SVR were 42.7% (38/89) in patients with HCV genotype 1b and 76.3% (45/59) in patients with HCV genotype 2 (2a or 2b). When virus-host DNA junctions from liver tissue were amplified, several bands were detected. Sequencing these PCR products revealed HBV integration in 54 of the 157 (34.4%) patients. HBV integration was detected in 34.1% (31/91) in patients with HCV genotype 1b and 34.8% (23/66) in patients with HCV genotype 2. In 4 of these 54 patients, multiple integration sites were present. HBV integration sites varied (Supplemental Digital Content, Table 1, http://links.lww.com/JCG/A80).

Integration of Hepatitis B Viral Genome and Response to Antiviral Therapy in Patients Infected With HCV Genotype 1b

HBV integration was detected in 31 of 91 patients (34.1%) infected with HCV genotype 1b. Baseline clinical characteristics were compared between patients with and without HBV integration in Table 2; no significant differences were observed, except for the percentage of patients who were positive for HBcAb. The virologic response 4 and 12 weeks after starting therapy and treatment outcome are

TABLE 1. Baseline Characteristics of All Study Patients (n = 157)

Age (y)	57.7 ± 10.2
Sex (male/female)	76 (48.4)/81 (51.6)
Body weight (kg)	58.5 ± 10.1
History of transfusion	32 (20.4)
Alanine aminotransferase (IU/L)	51.6 ± 46.9
Aspartate aminotransferase (IU/L)	45.7 ± 38.9
γ-glutamyl transpeptidase (IU)	50.9 ± 68.7
Alkaline phosphatase (IU/L)	263.1 ± 118.8
Albumin (g/dL)	4.15 ± 0.38
Total bilirubin (mg/dL)	0.65 ± 0.25
White blood cell count (/μL)	4962 ± 1354
Hemoglobin (g/dL)	14.0 ± 1.4
Platelet count ($\times 10^4$/μL)	179 ± 56
Liver histology—activity (A0/A1/A2/A3)	2 (1.3)/99 (63.0)/48 (30.6)/8 (5.1)
Liver histology—fibrosis (F0/F1/F2/F3)	3 (1.9)/112 (71.3)/32 (20.4)/10 (6.4)
HBV core antibody (negative/positive)	115 (73.2)/42 (26.8)
Pretreatment HCV RNA concentration ($\log_{10}$ IU/mL)	6.15 ± 0.54
HCV genotype (1b/2a/2b)	91 (58.0)/50 (31.8)/16 (10.2)
Genetic polymorphisms near the *IL28B* gene (TT/TG or GG)*	99 (78.6)/27 (21.4)
Treatment outcome (SVR/relapse/nonresponse)†	83 (56.1)/41 (27.7)/24 (16.2)

Percentages are shown in parentheses.
*Genetic polymorphism rs8099917. Genetic polymorphisms near the *IL28B* gene were not evaluated in 31 patients.
†Nine patients withdrew from the treatment.
HBV indicates hepatitis B virus; HCV, hepatitis C virus; NR, no response; SVR, sustained virologic response.

TABLE 2. Baseline Characteristics According to HBV Integration Status in Patients Infected With HCV Genotype 1b

	HBV Integration (+) n = 31	HBV Integration (−) n = 60	*P*
Age (y)	58.7 ± 8.4	59.2 ± 8.6	0.8602
Sex (male/female)	19 (61.3)/12 (38.7)	31 (51.7)/29 (48.3)	0.5127
Body weight (kg)	58.5 ± 9.3	57.7 ± 10.4	0.4359
Alanine aminotransferase (IU/L)	44.5 ± 25.9	58.6 ± 45.7	0.2477
Aspartate aminotransferase (IU/L)	50.9 ± 41.2	40.3 ± 36.9	0.1425
γ-glutamyl transpeptidase (IU)	50.0 ± 62.1	55.1 ± 50.0	0.3374
Alkaline phosphatase (IU/L)	232.5 ± 74.6	265.8 ± 101.3	0.1533
Albumin (g/dL)	4.10 ± 0.33	4.06 ± 0.41	0.8665
Total bilirubin (mg/dL)	0.65 ± 0.20	0.66 ± 0.25	0.9831
White blood cell count (/μL)	4908 ± 1436	4836 ± 1307	0.9266
Hemoglobin (g/dL)	14.1 ± 1.4	14.0 ± 1.2	0.8048
Platelet count ($\times 10^3$/μL)	164 ± 36	166 ± 56	0.7032
Liver histology—activity (A0/A1/A2/A3)	0/23 (74.2)/7 (22.6)/1 (3.2)	1 (1.7)/29 (48.3)/26 (43.3)/4 (6.7)	0.1230
Liver histology—fibrosis (F0/F1/F2/F3)	0/25 (80.7)/5 (16.1)/1 (3.2)	1 (1.7)/37 (61.7)/17 (28.3)/5 (8.3)	0.4318
HBV core antibody (negative/positive)	16 (51.6)/15 (48.4)	50 (83.3)/10 (16.7)	0.0030
Pretreatment HCV RNA concentration ($\log_{10}$ IU/mL)	6.13 ± 0.68	6.26 ± 0.41	0.8538
Genetic polymorphisms near the *IL28B* gene (TT/TG or GG)*	23 (85.2)/4 (14.8)	34 (70.8)/14 (29.2)	0.2631

Percentages are shown in parentheses.
*Genetic polymorphism rs8099917. Genetic polymorphisms near the *IL28B* gene were not measured in 16 patients.
HBV indicates hepatitis B virus; HCV, hepatitis C virus.

shown in Table 3. The percentage of patients who achieved RVR 4 weeks after starting therapy was 20.0% in patients with HBV integration and 5.1% in patients without HBV integration ($P = 0.0274$). In addition, the percentage of patients with $> 3 \log_{10}$ IU/mL reduction in HCV RNA levels, 4 weeks after starting therapy from pretreatment levels (including those achieving RVR) was significantly higher in patients with HBV integration than in those without HBV integration (63.3% vs. 35.6%; $P = 0.0129$). The percentage of patients who showed complete EVR 12 weeks after starting therapy was significantly higher among those with HBV integration than those without it (63.3% vs. 35.6%; $P = 0.0129$). In contrast, the percentage of patients who did not achieve EVR was significantly lower in patients with HBV integration than in those without (0% vs. 20.3%; $P = 0.0079$). With regard to treatment outcome, 63.3% of patients with HBV integration and 32.2% of patients without HBV integration achieved SVR. The SVR rate was significantly higher in patients with HBV integration ($P = 0.0050$).

Integration of Hepatitis B Viral Genome and Response to Antiviral Therapy in Patients Infected With HCV Genotype 2

In 66 patients infected with HCV genotype 2, HBV integration was detected in 23 patients (34.8%). No significant differences were observed in baseline clinical

TABLE 3. Virologic Response to Combination Therapy According to HBV Integration Status in Patients Infected With HCV Genotype 1b

	HBV Integration (+) n = 30	HBV Integration (−) n = 59
At 4 weeks		
Rapid virologic response (RVR)	6 (20.0)	3 (5.1)
Reduction $\geq 3 \log_{10}$ IU/mL	13 (43.3)	18 (30.5)
Reduction $\geq 2 \log_{10}$ IU/mL and $< 3 \log_{10}$ IU/mL	8 (26.7)	11 (18.6)
Reduction $\geq 1 \log_{10}$ IU/mL and $< 2 \log_{10}$ IU/mL	2 (6.7)	16 (27.1)
Reduction $< 1 \log$ IU/mL	1 (3.3)	11 (18.6)
At 12 weeks		
Complete early virologic response (cEVR)	19 (63.3)	21 (35.6)
Partial early virologic response (pEVR)	11 (36.7)	26 (44.1)
Nonearly virologic response (non-EVR)	0	12 (20.3)
Final outcomes		
Sustained virologic response (SVR)	19 (63.3)	19 (32.2)
Relapse	10 (33.3)	18 (30.5)
Nonresponse (partial response or null response)	1 (3.3)	22 (37.3)

Two patients withdrew from the treatment.
Percentages are shown in parentheses.
HBV indicates hepatitis B virus; HCV, hepatitis C virus.

TABLE 4. Univariate and Multivariate Analyses for Factors Associated With Sustained Virologic Response to Peginterferon and Ribavirin Combination Therapy

	Univariate Analysis	Multivariate Analysis	Odds Ratio (95% Confidence Interval)
Age (y)	0.1192	—	
Sex (male/female)	0.8368	—	
Body weight (kg)	0.6183	—	
Alanine aminotransferase (IU/L)	0.9133	—	
Aspartate aminotransferase (IU/L)	0.7943	—	
γ-glutamyl transpeptidase (IU)	0.3237	—	
Alkaline phosphatase (IU/L)	0.6469	—	
Albumin (g/dL)	0.0629	—	
Total bilirubin (mg/dL)	0.4122	—	
White blood cell count (/μL)	0.6354	—	
Hemoglobin (g/dL)	0.5245	—	
Platelet count ($\times 10^3$/μL)	0.0020	0.0306	19.690 (1.4251-331.31)
Liver histology—activity (A0-1/A2-3)	0.1653	—	
Liver histology—fibrosis (F0-1/F2-3)	0.0323	0.2573	0.5306 (0.1728-1.5770)
HBV core antibody (negative/positive)	0.1090		
Pretreatment HCV RNA concentration ($\log_{10}$ IU/mL)	0.1083		
HCV genotype (1b/2a or 2b)	0.0001	0.0003	5.6013 (2.2712-14.906)
Genetic polymorphisms near the *IL28B* gene (TT/TG or GG)	0.0468	0.0177	3.8013 (1.3123-12.166)
HBV integration into liver tissue DNA (positive/negative)	0.0161	0.0466	2.5623 (1.0311-6.6576)

HBV indicates hepatitis B virus; HCV, hepatitis C virus.

characteristics between patients with and without HBV integration, except for the percentage of patients who were positive for HBcAb (Supplemental Digital Content, Table 2, http://links.lww.com/JCG/A80). We found no significant differences in the rates of RVR ($P = 0.0531$), complete EVR ($P = 0.6245$), and SVR ($P = 0.6297$) between patients with and without HBV integration in patients infected with HCV genotype 2 (Supplemental Digital Content, Table 3, http://links.lww.com/JCG/A80).

Univariate and Multivariate Analyses for Factors Associated With SVR

Univariate and multivariate analyses were performed to identify factors that affect SVR (Table 4). Univariate analysis identified pretreatment platelet counts, the degree of liver fibrosis, HCV genotype, genetic polymorphisms near the *IL28B* gene, and the integration of HBV into the liver tissue as factors associated with SVR. Among these factors, pretreatment platelet counts, HCV genotype,

TABLE 5. Univariate and Multivariate Analyses for Factors Associated With Sustained Virologic Response to Peginterferon and Ribavirin Combination Therapy in Patients Infected With HCV Genotype 1b

	Univariate Analysis	Multivariate Analysis	Odds Ratio (95% Confidence Interval)
Age (y)	0.9225	—	
Sex (male/female)	0.2826	—	
Body weight (kg)	0.9129	—	
Alanine aminotransferase (IU/L)	0.8622	—	
Aspartate aminotransferase (IU/L)	0.4072	—	
γ-glutamyl transpeptidase (IU)	0.0840	—	
Alkaline phosphatase (IU/L)	0.0711	—	
Albumin (g/dL)	0.0730	—	
Total bilirubin (mg/dL)	0.3978	—	
White blood cell count (/μL)	0.9265	—	
Hemoglobin (g/dL)	0.4559	—	
Platelet count ($\times 10^3$/μL)	0.1004	—	
Liver histology—activity (A0-1/A2-3)	0.4351	—	
Liver histology—fibrosis (F0-1/F2-3)	0.1039	—	
HBV core antibody (negative/positive)	0.2559	—	
Pretreatment HCV RNA concentration ($\log_{10}$ IU/mL)	0.2559	—	
Genetic polymorphisms near the *IL28B* gene (TT/TG or GG)	0.0190	0.0389	9.3142 (1.6307-176.42)
HBV integration into liver tissue DNA (positive/negative)	0.0060	0.0320	3.1845 (1.1207-9.4392)

HBV indicates hepatitis B virus; HCV, hepatitis C virus.

genetic polymorphisms near the *IL28B* gene, and the integration of HBV into liver tissue were identified as independent factors associated with SVR by multivariate analysis.

When focusing on patients infected with HCV genotype 1b (Table 5), univariate analysis identified genetic polymorphisms near the *IL28B* gene and the integration of HBV into liver tissue as factors associated with SVR. These 2 factors were identified as independent factors associated with SVR by multivariate analysis.

DISCUSSION

In the present study, we detected HBV integration into the liver tissue in 34.4% of HBsAg-negative chronic hepatitis C patients. In 3 previous studies of HCV-related HCC, the rates of HBV integration in tumor tissue ranged from 55.6% (10 of 18 cases)[4] to 29.4% (10 of 34 cases)[6] and 0% (0 of 21 cases).[5] In cases of HBV integration into the HCC tumor tissue, clonal expansion of hepatocytes associated with the growth of HCC may account for discrepancies in the rates of HBV integration across studies. In contrast, clonal expansion of hepatocytes is unlikely in cases of integration into the liver tissue of patients with chronic hepatitis C. The prevalence of HBV integration in patients with chronic hepatitis C in our previous study was 23.1%,[15] which is comparable to the results from this study. Although no patients had a history of definite exposure to HBV, patients with HCV infection might have been at higher risk of HBV exposure than those without HCV infection, because both HCV and HBV are transfusion-transmissible viruses and the possible routes of transmission are similar.

In the present study, HBV integration sites were distributed across the genome, and the host sequences adjacent to the viral genome were divergent. HBV DNA has been reported to integrate randomly into the host DNA in cases of HBV-related HCC.[16–18] This random manner of HBV integration also appears in HBsAg-negative patients with chronic HCV infection.

Among patients infected with HCV genotype 1b, higher percentage of patients with HBV integration had a favorable viral response at 4 and 12 weeks of therapy and achieved SVR as treatment outcome. In addition, by multivariate analysis, HBV integration was an independent factor associated with SVR as well as genetic polymorphisms near the *IL28B* gene, the strongest factor associated with outcomes of PEG-IFN and ribavirin combination therapy in patients infected with HCV genotype 1.[19–23] The mechanism underlying the favorable viral responses in patients with the detection of HBV integration into the liver tissue is unknown. In case of HBsAg-positive HCC, in which HBV integration is detected in approximately 90% of patients,[16,24] integration could potentially cause the development of HCC in some patients by integrating adjacent to tumor-associated genes.[25,26] In the present study, HBV integration sites were adjacent to 5 potential IFN-related genes: *IRF8*, *TNFRSF10A*,[27] *SYN3*,[28] *REPS1*, and *SCARB1*[29] (Supplemental Digital Content, Table 1, http://links.lww.com/JCG/A80). These HBV integrations might have played a role in the viral response during PEG-IFN–based antiviral combination therapy. Moreover, X-region of HBV that was contained in integrated HBV genome in all cases might have affected the viral response, because this part has extensive trans-activating activity. However, such mechanisms have not been clarified. In addition, HBV integration sites in the majority of patients were not related to IFN-related genes, and other mechanisms will undoubtedly exist. These speculations are, therefore, far from satisfactory to explain the favorable response to antiviral therapy in patients with HBV integration. Further studies will be needed to elucidate the significance of HBV integration near IFN-related genes and to reveal underlying mechanisms.

In contrast to patients infected with HCV genotype 1b, no differences in viral response to combination antiviral therapy were found between HCV genotype 2-infected patients with and without HBV integration, except for a higher tendency of RVR rate. Given that the proportion of patients achieving RVR, complete EVR, and SVR were very high, HBV integration into liver tissue appears to have little impact on the response to antiviral therapy in patients with HCV genotype 2.

There are several limitations of this study. The detection of HBV integration with PCR using Alu repeats may limit the identification of HBV-X sequence integration sites that are far away from the priming site, thereby, restricting the sensitivity of the assay as the amplicon size increases. In addition, detection of HBV integration only using the X gene–specific primers makes it impossible to identify integration sites with other viral gene sequences. Further, the integrated HBV genome can limit or entirely negate the HBV-X primer-binding site asHBV sequences may be deleted upon integration. The Alu-PCR method used in the present study, therefore, may underestimate the integration of HBV in patients with chronic HCV infection. In addition, it was not clarified whether the integrated HBV fragment had potential transcriptional and translational function or truncated nonfunction fragment. The evaluations of the translated proteins in liver tissue will be needed for the confirmation. Finally, the sample size of study patients was not large enough to lead the solid conclusion on the effect of HBV integration on the response to the antiviral combination therapy with PEG-IFN and ribavirin for HCV infection.

In summary, HBV integration was detected in 54 of the 157 patients with chronic hepatitis C. Integrated sites were distributed across the genome. HBV integration was associated with favorable viral responses to combination therapy with PEG-IFN and ribavirin, including the treatment outcome in patients with HCV genotype 1b. Although the underlying mechanisms are unclear, the results of this observational study will encourage the investigation on the significance of HBV integration in the management of HCV-infected HBsAg-negative patients. Further studies with larger study populations and methods more sensitive and reliable than Alu-PCR for the detection of HBV integration are needed to elucidate the association between HBV integration and response to combination therapy in patients with chronic hepatitis C.

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Research Article

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Effect of nucleos(t)ide analogue therapy on hepatocarcinogenesis in chronic hepatitis B patients: A propensity score analysis

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Background & Aims: Some patients with chronic hepatitis B virus (HBV) infection progress to hepatocellular carcinoma (HCC). However, the long-term effect of nucleos(t)ide analogue (NA) therapy on progression to HCC is unclear.

Methods: Therefore, we compared chronic hepatitis B patients who received NA therapy to those who did not, using a propensity analysis.

Results: Of 785 consecutive HBV carriers between 1998 and 2008, 117 patients who received NA therapy and 117 patients who did not, were selected by eligibility criteria and propensity score matching. Factors associated with the development of HCC were analyzed. In the follow-up period, HCC developed in 57 of 234 patients (24.4%). Factors significantly associated with the incidence of HCC, as determined by Cox proportional hazards models, include higher age (hazard ratio, 4.36 [95% confidence interval, 1.33–14.29], $p = 0.015$), NA treatment (0.28 [0.13–0.62], $p = 0.002$), basal core promoter (BCP) mutations (12.74 [1.74–93.11], $p = 0.012$), high HBV core-related antigen (HBcrAg) (2.77 [1.07–7.17], $p = 0.036$), and high gamma glutamyl transpeptidase levels (2.76 [1.49–5.12], $p = 0.001$).

Conclusions: NA therapy reduced the risk of HCC compared with untreated controls. Higher serum levels of HBcrAg and BCP mutations are associated with progression to HCC, independent of NA therapy.

Introduction

An estimated 350 million individuals worldwide are chronically infected with hepatitis B virus (HBV), of whom 1 million die annually from HBV-related liver disease [1]. Chronic HBV infection is recognized as a major risk factor for the development of hepatocellular carcinoma (HCC) [1,2]. Hepatitis B surface antigen (HBsAg)-positive patients have a 70-fold increased risk of developing HCC compared to HBsAg seronegative counterparts [3,4]. HBV infection is endemic in Southeast Asia, China, Taiwan, Korea, and sub-Saharan Africa, where up to 85–95% of patients with HCC are HBsAg positive [5]. HCC is the third and fifth leading cause of cancer death in men and women, respectively, and the number of deaths and the mortality rate from HCC have greatly increased in Japan since 1975 [6]. Hepatitis C virus (HCV)-related HCC accounts for 75% of all HCCs in Japan and HBV-related HCC accounts for 15% [6].

In 2004, Liaw *et al.* reported a significant reduction in HCC in 651 adults receiving lamivudine after adjustment for baseline variables (hazard ratio, 0.49 [95% confidence interval (95% CI), 0.25–0.99], $p = 0.047$) [7]. However, the results were not significant after exclusion of 5 patients who developed HCC within 1 year of randomization (0.47 [0.22–1.00], $p = 0.052$). Therefore, in 2009, the National Institutes of Health Consensus Development Conference concluded that there was insufficient evidence to assess whether nucleos(t)ide analogue (NA) therapy can prevent the development of HCC [8].

The long-term use of lamivudine has not been recommended because of tyrosine–methionine–aspartate–aspartate (YMDD) mutations, which have occasionally been associated with severe and even fatal flares of hepatitis [9,10]. Therefore, adefovir dipivoxil should be added immediately in patients with virological or biochemical breakthroughs or no response. Currently, there are 2 nucleoside agents (lamivudine, entecavir) and 1 nucleotide agent (adefovir dipivoxil) available for treatment of HBV infection in Japan. The agent with the higher genetic barrier to resistance, entecavir, is considered the initial drug of choice [11]. Recently, 3 studies on lamivudine suggested that long-term sustained viral suppression was associated with a reduced likelihood of developing HCC [12–14].

In this study, we sought to determine if NA therapy was associated with a reduction in the development of HCC. Since the validity of treatment effects in observational studies may be limited by selection bias and confounding factors, we performed a propensity analysis [15].

Keywords: HBcrAg; BCP; Gamma-GTP; Average integration value; HBV DNA.
Received 25 July 2012; received in revised form 17 October 2012; accepted 22 October 2012; available online 30 October 2012

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Abbreviations: HCC, hepatocellular carcinoma; HBV, hepatitis B virus; NA, nucleos(t)ide analogue; HBcrAg, HBV core-related antigen; BCP, basal core promoter; gamma-GTP, gamma glutamyl transpeptidase.

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Materials and methods

Patient selection

The study protocol was approved by the Institutional Ethics Committee of Ogaki Municipal Hospital in January 2011, and was in compliance with the Declaration of Helsinki. Written informed consent for the use of stored serum samples for the study was obtained from all patients.

Between 1998 and 2008, 1220 consecutive HBsAg-positive patients, who visited the Department of Gastroenterology and Hepatology at Ogaki Municipal Hospital, were prospectively enrolled in our HCC surveillance program. Of these, 785 patients met the following inclusion criteria: HBsAg positive for more than 6 months, no evidence of HCV co-infection, exclusion of other causes of chronic liver disease (alcohol consumption >80 g/day, hepatotoxic drugs, autoimmune hepatitis, primary biliary cirrhosis, hemochromatosis, or Wilson's disease), follow-up duration of greater than 3 years, no evidence of HCC for at least 1 year from the start of the follow-up period, receiving no interferon treatment, and receiving NA therapy for more than 1 year before the detection of HCC (Fig. 1). In patients on NA therapy, the date of NA therapy initiation was considered the starting point of the follow-up period.

Of these 785 patients, 148 received NA therapy (NA group) and 637 patients did not receive NA therapy (non-NA group) during the follow-up period. To reduce the confounding effects of covariates, we used propensity scores to match NA patients to unique non-NA patients. Six covariates including age, sex, HBV DNA concentration, hepatitis B e antigen (HBeAg), platelet count, and alanine aminotransferase (ALT) activity were taken into account at the start of follow-up. We computed the propensity score by using logistic regression with the independent variable including age (⩽40 years or >40 years), sex (female or male), HBV DNA concentration (⩽5.0 log copies/ml or >5.0 log copies/ml), HBeAg (negative or positive), platelet count (>150 × 10^3/m^3 or ⩽150 × 10^3/m^3), and ALT activity (⩽40 IU/ml or >40 IU/ml), as shown in previous reported cut-off values according to the indication for NA therapy [16–19]. This model yielded a *c* statistic of 0.85 (95% confidence interval [CI], 0.82–0.88), indicating very good ability of the propensity score model to predict treatment status. We sought to match each patient who received NA therapy to a patient who did not receive NA therapy, having a propensity by using greedy 5–1 digit matching [20]. Once this threshold was exceeded, a patient with NA therapy was excluded. This score ranged from 0.09198 to 0.98967 and, in effect, represented the probability that a patient would be receiving NA. We were able to match 117 patients with NA therapy to 117 unique patients without NA therapy. The follow-up period ended on 31 December, 2011 or the date when HCC occurrence was identified.

Surveillance and diagnosis

All patients were followed up at our hospital at least every 6 months. During each follow-up examination, platelet count, ALT, gamma glutamyl transpeptidase (gamma-GTP), total bilirubin, alkaline phosphatase (ALP), albumin, and alpha-fetoprotein (AFP) levels were measured. We used commercially available kits to test blood samples for HBsAg, HBeAg, and anti-HBe (Abbott Japan Co., Ltd., Tokyo, Japan). Before November 2007, the serum HBV DNA concentration was monitored by a polymerase chain reaction assay (COBAS Amplicor HBV monitor test, Roche Diagnostics K. K., Tokyo, Japan) with a lower detection limit of approximately 2.6 log copies/ml, and after December 2007, it was monitored with another polymerase chain reaction assay (COBAS AmpliPrep-COBAS TaqMan HBV Test, Roche Diagnostics K. K.), with a lower detection limit of approximately 2.1 log copies/ml. HBV genotyping was performed as described previously [21]. Serum levels of HBV core-related antigen (HBcrAg) were measured using a chemiluminescence enzyme immunoassay (CLEIA) as described previously [22,23]. Precore nucleotide 1896 and basal core promoter (BCP) dinucleotide 1762/1764 were determined using the line probe assay (INNO-LiPA HBV PreCore assay; Innogenetics NV) [24,25]. The probes were designed to determine the nucleotides at position 1896 (G *vs.* A) in the precore region and positions 1762 (A *vs.* T) and 1764 (G *vs.* A and G *vs.* T) in the BCP region. A line probe assay was used to identify any emergence of YMDD mutations (INNO-LiPA HBV DR assay; Innogenetics NV).

Platelet count, ALT, gamma-GTP, total bilirubin, ALP, albumin, AFP, and HBV DNA values were expressed as average integration values [26,27] after the start of follow-up.

According to the Clinical Practice Guidelines for Hepatocellular Carcinoma in Japan [28], we performed ultrasound (US) and monitoring of 3 biomarkers (AFP, *Lens culinaris* agglutinin-reactive fraction of alpha-fetoprotein [AFP-L3], and des-gamma-carboxy prothrombin [DCP]) every 3–4 months, and dynamic magnetic resonance imaging (MRI) every 12 months, for patients with cirrhosis under surveillance. For patients with chronic hepatitis, we performed US and monitoring of the 3 biomarkers every 6 months. Histological examinations were performed in 91 out of 234 patients. Among them, cirrhosis was diagnosed in 32 patients. In the remaining 143 patients, the diagnosis of cirrhosis was made according to typical US findings, e.g., superficial nodularity, a coarse parenchymal echo pattern, and signs of portal hypertension (splenomegaly >120 mm, dilated portal vein diameter >12 mm, patent collateral veins, or ascites) [29–31]. Patients who did not satisfy these criteria were classified as having chronic hepatitis. One hundred and forty-two patients were diagnosed with chronic hepatitis and 92 patients with cirrhosis. For diagnostic confirmation of HCC, patients underwent dynamic MRI. A histological diagnosis of HCC was made in 28 patients (surgical specimen, 23 patients; US-guided needle biopsy specimen, 5 patients). The remaining 29 patients were diagnosed with HCC based on typical dynamic MRI findings, including hypervascularity in the arterial phase with washout in the portal venous or delayed phase [32].

Treatments

In the NA group, 117 patients received NA therapy including 18 patients with lamivudine, 28 patients with lamivudine and adefovir dipivoxil, and 71 patients with entecavir. The indications for NA therapy followed the guidelines of the American Association for the Study of Liver Diseases (AASLD), the European Association for the Study of the Liver (EASL), or the Asian Pacific Association for the Study of the Liver (APASL) [33–35]. In contrast, of the 117 patients not on NA therapy, 104 did not receive treatment before NA was not yet approved in Japan and the remaining 13 patients declined NA therapy.

Statistical analysis

Continuous variables are expressed as medians (range). The Mann–Whitney *U* test was used for continuous variables, and the Chi-square test with Yates' correction or Fisher's exact test was used for categorical variables. Actuarial analysis of the cumulative incidence of hepatocarcinogenesis was performed using the Kaplan–Meier method, and differences were tested with the log-rank test. The Cox proportional hazards model and the forward selection method were used to estimate the relative risk of HCC associated with age (⩽40 years or >40 years), sex (female or male), treatment (NA or no NA), HBsAg (⩽3.0 log IU/ml or >3.0 log IU/ml), HBV DNA level (⩽5.0 log copies/ml or >5.0 log copies/ml), HBeAg (negative or positive), precore region (wild type or mutant), BCP (wild type or mutant type), HBcrAg (⩽3.0 logU/ml or >3.0 logU/ml), platelet count (>150 × 10^3/m^3 or ⩽150 × 10^3/m^3), ALT (⩽40 IU/ml or >40 IU/ml), total bilirubin, gamma-GTP, ALP, albumin, and AFP (⩽10 ng/ml or >10 ng/ml) for univariate and multivariate analyses. We used the minimum or maximum of the reference values at our institution as cut-off values for total bilirubin, gamma-GTP, ALP, and albumin. We conducted a sensitivity analysis to determine the magnitude of an unmeasured confounder [36].

We considered *p* values of 0.05 or less to be significant. Statistical analysis was performed with SPSS, version 18.0 for Windows (International Business Machines Corporation, Tokyo, Japan).

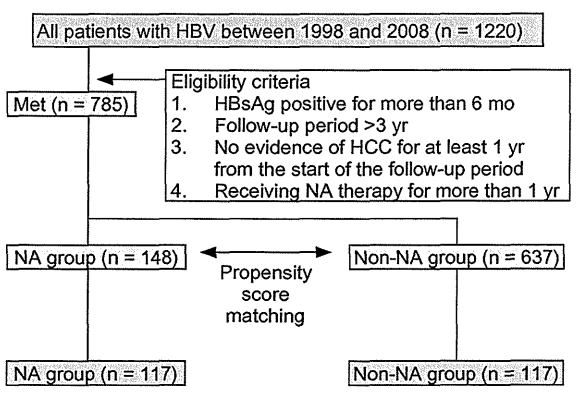

Fig. 1. Flowchart of the patient selection process.

Table 1. Baseline characteristics of all patients.

	NA group (n = 148)	Non-NA group (n = 637)	*p* value	Standardized difference in %
Age (yr)	53 (26-81)	48 (4-85)	<0.0001	40.6
Sex (female/male)	60/88	285/352	0.5378	6.1
Genotype (A/B/C/D/F/n.d.)	2/5/137/0/1/2	24/60/389/2/0/162	<0.0001	37.6
HBsAg (log_{10} IU/ml)	3.5 (-0.1-5.5)	3.3 (-1.3-7.9)	<0.0001	53.8
HBV DNA (log_{10} copies/ml)	7.0 (2.6-9.6)	3.8 (2.3-9.9)	<0.0001	99.9
HBeAg (±)	76/72	151/486	<0.0001	62.8
Precore region (W/M/n.d.)	30/109/9	88/381/168	0.4652	0.0
BCP (W/M/n.d.)	33/123/10	135/279/205	0.0074	27.3
HBcrAg (log_{10} U/ml)	5.9 (2.9-7.0)	3.0 (2.9-7.0)	<0.0001	96.7
Platelet count (x10^3/m^3)	150 (32-388)	188 (37-503)	<0.0001	-59.7
ALT (IU/ml)	65 (7-1088)	26 (5-3410)	<0.0001	44.1
AFP (ng/ml)	3.9 (0.8-3363)	2.9 (0.8-3686)	0.0062	-6.2
Cirrhosis (presence/absence)	62/86	91/546	<0.0001	59.1
Child-Pugh classification (A/B)	132/16	618/19	0.0002	32.7
Follow-up duration (yr)	12.8 (3.1-19.6)	13.7 (3.1-20.0)	0.1565	-16.9
Administration period (yr)	6.5 (1.5-11.0)	-	-	-
Propensity score	0.58093 (0.09198-0.98686)	0.95253 (0.12913-0.98967)	<0.0001	-132.3

NA, nucleos(t)ide analogue; n.d., not done; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HBeAg, hepatitis B e antigen; W, wild type; M, mutant type; BCP, basal core promoter; HBcrAg, hepatitis B core-related antigen; ALT, alanine aminotransferase; AFP, alpha-fetoprotein; Child-Pugh classification, reference no [50], Standardized difference in%; $100(X_{NA} - X_{non\text{-}NA})/([S^2_{NA} + S^2_{non\text{-}NA}]/2)^{1/2}$, where for each covariate X_{NA} and $X_{non\text{-}NA}$ are the sample means in NA and non-NA groups, respectively, and S^2_{NA} and $S^2_{non\text{-}NA}$ are the corresponding sample variances.

Results

Patient characteristics

Table 1 shows baseline characteristics of all 785 patients before propensity matching. There were significant differences in age, HBV genotype, HBsAg, HBV DNA concentration, presence of HBeAg, BCP mutations, HBcrAg, platelet counts, ALT level, AFP level, presence of cirrhosis, and Child-Pugh classification. The baseline characteristics of the 234 study patients after propensity matching are summarized in Table 2. There are no significant differences in age, sex, HBV genotype, HBsAg, HBV DNA concentration, presence of HBeAg, precore region mutations, BCP mutations, platelet counts, ALT concentration, Child-Pugh classification, and follow-up duration. HBcrAg concentration was significantly higher in the NA group than in the non-NA group. NA was administered a median of 6.1 years (range: 1.5–10.7 years).

Factors associated with the incidence of hepatocarcinogenesis

Factors associated with the incidence of HCC as determined by the Cox proportional hazard models and the forward selection method were analyzed in all 785 patients. High age (hazard ratio, 6.43 [95% CI, 2.71–15.26], p <0.001), male sex (3.43 [1.67–7.02], p = 0.002), NA treatment (0.28 [0.21–0.85], p = 0.017), BCP mutation (19.96 [2.27–141.90], p = 0.03), high HBcrAg levels (8.21 [3.40–19.85], p <0.001), and high AFP levels (2.49 [1.43–4.34], p = 0.001) were significantly associated with the incidence of HCC.

HCC developed in 57 of 234 patients (24.4%) during follow-up after propensity matching. The 5-year, 7-year, and 10-year cumulative incidences of HCC were 9.6%, 20.4%, and 33.4%, respectively. The 5-year, 7-year, and 10-year cumulative incidences of HCC were 2.7%, 3.3%, and 3.3%, respectively, in patients on NA therapy (n = 117) and 11.3%, 26.0%, and 40.0% in patients not on NA therapy (n = 117). Hepatocarcinogenesis occurred at significantly higher rates in the non-NA group (p = 0.0094, Fig. 2). The 5-year, 7-year, and 10-year cumulative incidences of HCC were 0.0%, 0.0%, and 0.0%, respectively, in patients with wild type BCP (n = 38) and 11.0%, 25.2%, and 41.9% in patients with mutant BCP (n = 112; p = 0.0006, Fig. 3). Factors associated with the incidence of HCC as determined by the Cox proportional hazard models and the forward selection method are listed in Table 3. Higher age (hazard ratio, 4.36 [95% CI, 1.33–14.29], p = 0.015), NA treatment (0.28 [0.13–0.62], p = 0.002), BCP mutation (12.74 [1.74–93.11], p = 0.012), high HBcrAg levels (2.77 [1.07–7.17], p = 0.036), and high gamma-GTP levels (2.76 [1.49–5.12], p = 0.001) were significantly associated with the incidence of HCC. In addition, 2 patients died due to hepatic failure during the follow-up period in the non-NA group.

The sensitivity analysis found that the observed relationship between NA treatment and HCC incidence could be diminished by the unmeasured confounder that the high prevalence of the unmeasured confounder is greater in the non-NA group than in the NA group. For example, suppose a binary unmeasured confounder that increased the hazard of HCC incidence (hazard ratio, 1.50) was present in 40% of those who were treated with NA and 80% of those who were not treated with NA. Then, the study's result would become less extreme and would no longer be statistically significant (hazard ratio under sensitivity analysis, 0.48 [95% CI, 0.22–1.05]).

Follow-up data of various parameters in patients on or not on NA therapy

For this analysis, we used the average integration value during the follow-up period (Table 4). ALT, gamma-GTP, ALP, AFP, and

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Table 2. Baseline characteristics of patients on NA therapy and propensity-matched controls.

	NA group (n = 117)	Non-NA group (n = 117)	*p* value	Standardized difference in %
Age (yr)	52 (27-77)	52 (21-77)	0.9223	1.7
Sex (female/male)	44/73	45/72	0.8929	6.1
Genotype (A/B/C/n.d.)	1/4/109/3	4/7/85/21	0.1232	26.8
HBsAg (log_{10} IU/ml)	3.6 (0.9-5.5)	3.6 (0.9-7.9)	0.1440	29.9
HBV DNA (log_{10} copies/ml)	6.7 (2.6-9.6)	6.5 (2.3-9.6)	0.1273	20.5
HBeAg (±)	57/60	58/59	0.8960	2.0
Precore region (W/M/n.d.)	22/87/8	16/75/26	0.6399	5.1
BCP (W/M/n.d.)	22/88/7	17/70/30	0.9359	0.0
HBcrAg (log_{10} U/ml)	5.9 (2.9-7.0)	4.9 (2.9-7.0)	0.0022	41.2
Platelet count ($x10^3/m^3$)	143 (32-262)	146 (37-396)	0.6340	-12.1
ALT (IU/ml)	68 (7-1088)	55 (9-3410)	0.0977	1.9
AFP (ng/ml)	2.8 (0.8-402)	3.9 (0.8-1010)	0.3118	-13.5
Cirrhosis (presence/absence)	48/69	44/73	0.6882	6.1
Child-Pugh classification (A/B)	108/9	104/13	0.5024	3.1
Follow-up duration (yr)	12.3 (3.1-19.4)	11.6 (3.1-18.3)	0.7346	-4.5
Administration period (yr)	6.1 (1.5-10.7)	-	-	-
Propensity score	0.65895 (0.11449-0.96977)	0.65895 (0.12913-0.96989)	0.9931	0.0

NA, nucleos(t)ide analogue; n.d., not done; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HBeAg, hepatitis B e antigen; W, wild type; M, mutant type; BCP, basal core promoter; HBcrAg, hepatitis B core-related antigen; ALT, alanine aminotransferase; AFP, alpha-fetoprotein; Child-Pugh classification, reference no [50], Standardized difference in%; $100(X_{NA} - X_{non\text{-}NA})/([s^2_{NA} + s^2_{non\text{-}NA}]/2)^{1/2}$, where for each covariate X_{NA} and $X_{non\text{-}NA}$ are the sample means in NA and non-NA groups, respectively, and s^2_{NA} and $s^2_{non\text{-}NA}$ are the corresponding sample variances.

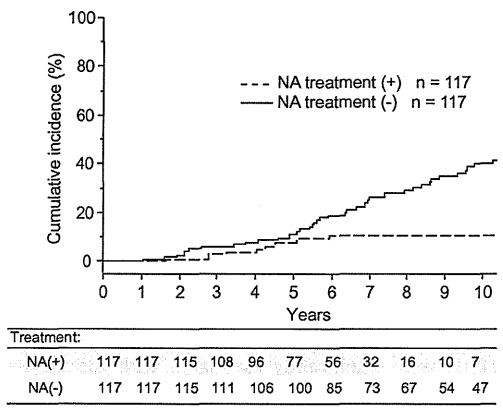

Fig. 2. Incidence of hepatocellular carcinoma (HCC) according to nucleos(t)ide analogue (NA) treatment status. The NA group had a significantly higher rate of progression to HCC than the non-NA group ($p = 0.0094$).

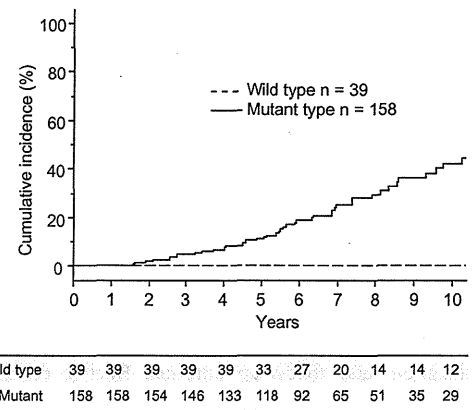

Fig. 3. Incidence of hepatocellular carcinoma (HCC) according to basal core promoter (BCP) mutations. Patients with mutant-type BCP had a significantly higher rate of progression to HCC than those with wild type BCP ($p = 0.0006$).

HBV DNA levels were significantly lower in patients on NA therapy than in patients not on NA therapy. In contrast, platelet counts and albumin levels were significantly higher in patients on NA therapy than in patients not on NA therapy.

Discussion

Our study shows that long-term NA maintenance therapy is associated with the suppression of progression to HCC. Liaw *et al.* reported that lamivudine decreased the risk of HCC in cirrhotic patients [7]. However, it is unclear whether the observed decreased risk of HCC with NA therapy was due to the short observation period in their study. It is very difficult to prove the preventive effect of NA on the development of HCC, because randomized control studies are not ethically possible. In this study, patients on NA therapy were compared to propensity score-matched untreated controls. In these control patients, NA therapy had not yet been approved or was not routinely used for chronic hepatitis B at the time, or was declined by the patient. As opposed to the entire population, these propensity-matched patients were well matched to patients on NA; significant differences included higher HBcrAg levels in the NA group.

Large community-based studies have confirmed that advanced age, male sex, HBeAg positivity, low platelet count,

Table 3. Factors associated with progression to hepatocellular carcinoma among propensity-matched patients (Cox proportional hazard model).

		Adjusted hazard ratio (95% CI)	*p* value
Age (yr)	≤40	1	0.015
	>40	4.36 (1.33-14.29)	
Treatment	no NA	1	0.002
	NA	0.28 (0.13-0.62)	
BCP	wild-type	1	0.012
	mutant-type	12.74 (1.74-93.11)	
HBcrAg (log_{10} U/ml)	≤3.0	1	0.036
	>3.0	2.77 (1.07-7.17)	
γ-GTP (IU/L)	≤56	1	0.001
	>56	2.76 (1.49-5.12)	

NA, nucleos(t)ide analogue; BCP, basal core promoter; HBcrAg, hepatitis B core-related antigen; γ-GTP, gamma glutamyl transpeptidase.

higher ALT levels, elevated AFP levels, and presence of cirrhosis are factors associated with the development of cirrhosis and HCC [17,18]. Platelet count is a useful surrogate marker for the diagnosis of cirrhosis [37]. All subjects were not histologically diagnosed in this study. Therefore, we selected platelet count as a marker of hepatic fibrosis instead of cirrhosis. An elevated ALT level indicates the presence of active disease, and persistently elevated AFP levels are a reflection of an enhanced regenerative state in the liver [16]. In the REVEAL study, a high HBV DNA load was associated with an increased rate of HCC development [17]. A direct correlation was observed between baseline HBV DNA levels and the incidence of HCC, independent of serum ALT concentration. In a model that integrated baseline and follow-up HBV DNA levels, the cumulative incidence of HCC ranged from 1.3% in patients with undetectable levels of HBV DNA to 14.9% in patients with HBV DNA levels greater than or equal to 10^6 copies/ml. Therefore, we have selected factors, such as age, sex, HBeAg serostatus, HBV DNA concentration, platelet count, and ALT for propensity matching.

Although the exact mechanisms of hepatocarcinogenesis by HBV remain unclear, two mechanisms have been proposed [38,39]. One mechanism involves chronic necroinflammation of hepatocytes, cellular injury, and hepatocyte regeneration [40]. The other mechanism involves the direct carcinogenicity of HBV through chromosomal integration [41]. Complete and sustained viral suppression by NA might block both pathways and prevent the development of HCC. It is well known that the rate of HCC is significantly higher in patients with virological breakthrough or no response. In our study, when virological or biochemical breakthrough was observed and the YMDD mutation was detected in patients on lamivudine, adefovir dipivoxil was immediately added. In patients with cirrhosis, especially in the decompensated stage, sustained viral response on NA therapy was not necessarily associated with a preventative effect against the development of HCC, even though the incidence was lower than in a group not on NA [14]. It is not surprising that viral suppression decreased but did not eliminate the risk of HCC, because HBV DNA may have already integrated into the host genome before the initiation of therapy and may have resulted in genomic alternations, chromosomal instability, or both [42,43].

It is reported that patients with HBV genotype C infection have higher HBV DNA levels, higher frequency of pre-S deletions, higher prevalence of BCP T1762/A1764 mutations, and significantly higher chances of developing HCC [16,44–46]. In our study, T1762/A1764 mutations were observed in 158 (80.2%) out of 197 patients and were associated with a higher risk of developing HCC (adjusted hazard ratio, 12.740 [95% CI 1.743–93.108]), independent of NA therapy. However, the BCP T1762/A1764 mutations were detected in HCC patients from Asia and Africa, where HBV genotype C infection is predominant [16].

HBcrAg is a new HBV marker that reflects HBV load and corresponds to HBV DNA levels [21]. HBcrAg is comprised of HBV core antigen (HBcAg) and HBeAg; both are products of the pre-core/core gene and share the first 149 amino acids of HBcAg. The HBcrAg assay measures HBcAg and HBeAg simultaneously by using monoclonal antibodies that recognize both denatured HBcAg and HBeAg [47]. Serum HBcrAg concentration is well correlated with intrahepatic levels of covalently closed circular DNA (cccDNA) [48]. It is reported that HBcrAg is a useful marker for guiding cessation of NA therapy and evaluation of disease activity [21,49]. In our study, elevated serum HBcrAg concentration was associated with a higher risk of developing HCC (adjusted hazard ratio, 2.767 [95% CI 1.067–7.172]). This is the first report demonstrating a relationship between HBcrAg and HCC.

The present study has several limitations. The retrospective design might have introduced an unintended bias. The propensity matching method was adopted to reduce the confounding effects of covariates. Characteristics of patients who did or did not receive NA therapy were similar except for HBcrAg concentration.

Table 4. Average integration values of various parameters in patients who did or did not receive NA therapy.

	NA group (n = 117)	Non-NA group (n = 117)	*p* value
Platelet count (x10^3/m^3)	17.0 (3.3-37.2)	14.8 (3.3-296)	0.0060
ALT (IU/ml)	28.2 (8.5-88.9)	39.1 (12.2-737.5)	<0.0001
γ-GTP (IU/L)	27.0 (10.9-267.6)	36.2 (9.5-269.7)	0.0427
Total bilirubin (mg/dl)	0.7 (0.3-2.0)	0.7 (0.3-2.6)	0.1554
ALP (IU/L)	242.7 (113.5-1028.8)	265.2 (140.5-1247.6)	0.0127
Albumin (g/dl)	4.4 (3.0-5.0)	4.0 (2.4-4.8)	<0.0001
Alpha-fetoprotein (ng/ml)	2.2 (0.8-106.0)	4.5 (0.9-723.8)	<0.0001
HBV DNA (log_{10} copies/ml)	2.5 (2.1-8.9)	4.6 (2.1-9.3)	<0.0001

NA, nucleos(t)ide analogue; ALT, alanine aminotransferase; γ-GTP, gamma glutamyl transpeptidase; ALP, alkaline phosphatase; HBV, hepatitis B virus.

Research Article

However, the non-NA group included many historical cases when NA therapy was not yet available. In addition, the HBV DNA assay used between 1998 and 2007 was not the most sensitive one.

In conclusion, NA therapy reduces the risk of HCC compared with untreated controls. Higher serum HBcrAg levels and BCP mutations are associated with development of HCC, independent of NA therapy.

Financial support

This work was supported by Health and Labour Sciences Research Grants (Research on Hepatitis) from the Ministry of Health, Labour and Welfare of Japan.

Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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Cytoglobin is expressed in hepatic stellate cells, but not in myofibroblasts, in normal and fibrotic human liver

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Cytoglobin (CYGB) is ubiquitously expressed in the cytoplasm of fibroblastic cells in many organs, including hepatic stellate cells. As yet, there is no specific marker with which to distinguish stellate cells from myofibroblasts in the human liver. To investigate whether CYGB can be utilized to distinguish hepatic stellate cells from myofibroblasts in normal and fibrotic human liver, human liver tissues damaged by infection with hepatitis C virus (HCV) and at different stages of fibrosis were obtained by liver biopsy. Immunohistochemistry was performed on histological sections of liver tissues using antibodies against CYGB, cellular retinol-binding protein-1 (CRBP-1), α-smooth muscle actin (α-SMA), thymocyte differentiation antigen 1 (Thy-1), and fibulin-2 (FBLN2). CYGB- and CRBP-1-positive cells were counted around fibrotic portal tracts in histological sections of the samples. The expression of several of the proteins listed above was examined in cultured mouse stellate cells. Quiescent stellate cells, but not portal myofibroblasts, expressed both CYGB and CRBP-1 in normal livers. In fibrotic and cirrhotic livers, stellate cells expressed both CYGB and α-SMA, whereas myofibroblasts around the portal vein expressed α-SMA, Thy-1, and FBLN2, but not CYGB. Development of the fibrotic stage was positively correlated with increases in Sirius red-stained, α-SMA-positive, and Thy-1-positive areas, whereas the number of CYGB- and CRBP-1-positive cells decreased with fibrosis development. Primary cultured mouse stellate cells expressed cytoplasmic CYGB at day 1, whereas they began to express α-SMA at the cellular margins at day 4. Thy-1 was undetectable throughout the culture period. In human liver tissues, quiescent stellate cells are CYGB positive. When activated, they also become α-SMA positive; however, they are negative for Thy-1 and FBLN2. Thus, CYGB is a useful marker with which to distinguish stellate cells from portal myofibroblasts in the damaged human liver.
Laboratory Investigation (2014) **94,** 192–207; doi:10.1038/labinvest.2013.135; published online 2 December 2013

KEYWORDS: α-smooth muscle actin; cellular retinol binding protein-1; chronic hepatitis; cytoglobin; fibulin-2; Thy-1

Stellate cell activation-associated protein was originally discovered by proteomic analysis (in 2001)[1] in cultured rat hepatic stellate cells that have vitamin A storage ability when quiescent and function as liver-specific pericytes. Histoglobin[2] and Cytoglobin (CYGB)[3] were reported by Trent and Hargrove[2] and by Burmester *et al*,[3] respectively, in 2002. These proteins, in addition to stellate cell activation-associated protein, were classified as human, mouse, and rat homologs of a hexacoordinate globin that differs from the traditional pentacoordinate globins, such as myoglobin and hemoglobin.[4] CYGB consists of 190 amino acids with a calculated molecular mass of 21 kDa, and its amino acid sequence is highly conserved among species.[3] Human CYGB has ~25% amino acid identity with vertebrate myoglobin and hemoglobin and 16% identity with human neuroglobin, which is another type of globin that is present specifically in the nervous system. CYGB is thus recognized as the fourth globin of mammals.[5] The *CYGB* gene is located on human chromosome 17q25.3 and mouse chromosome 11E2.

Although myoglobin, hemoglobin, and neuroglobin are tissue restricted to cardiomyocytes and skeletal myofibers, erythrocytes, and the nervous system, respectively, CYGB is ubiquitously expressed in the cytoplasm of mesenchymal fibroblastic cells of many organs,[6] and CYGB was reported to

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Received 26 April 2013; revised 20 September 2013; accepted 4 October 2013

be present in the nucleus of human hepatocytes.[7] In particular, CYGB is present in stellate cells in the liver and pancreas, reticulocytes in the spleen, mesenchymal cells in the submucosal layer of the gut, and mesangium cells and stromal cells in the rat kidney. Therefore, one interesting aspect of CYGB expression is its presence in visceral cells that have the ability to store vitamin A. CYGB has also been observed in some neuronal subpopulations of the central and peripheral nervous systems in humans.

Hepatic stellate cells have conventionally been recognized as hepatic fibroblastic cells (myofibroblasts are also categorized as this cell type) that preferentially localize to the portal region. Considering that quiescent stellate cells are transformed into activated stellate cells, the liver contains at least three types of fibroblastic cells: stellate cells, activated stellate cells, and portal myofibroblasts.[8,9] Stellate cells are desmin positive in rodents[10] and, when activated, they express α-smooth muscle actin (α-SMA). In addition, these cells express cellular retinol-binding protein-1 (CRBP-1) and participate in the metabolism of retinol and retinyl esters.[11] In contrast, thymocyte differentiation antigen 1 (Thy-1 or CD90)[12–14] and fibulin-2 (FBLN2)[15–17] have been utilized as markers of liver myofibroblasts. These cell type-specific markers of liver fibroblastic cells have been largely utilized in studies with rodents. However, a specific marker to distinguish stellate cells from myofibroblasts in the human liver has not yet been identified.

The aims of our present work were to investigate whether CYGB is a reliable marker of stellate cells in the normal human liver and to study the expression of CYGB, CRBP-1, Thy-1, FBLN2, and α-SMA in fibrotic and cirrhotic human liver.

MATERIALS AND METHODS

Human Liver Tissues

Human liver tissues damaged by hepatitis C virus (HCV) infection at various fibrosis stages (from F1 to F4, 10 samples each) and one tissue sample damaged by nonalcoholic steatohepatitis (NASH) at fibrosis stage F2 (58-year-old woman with serum alanine aminotransferase (ALT) 110 IU/l) were obtained by liver biopsy at Osaka City University Medical School Hospital (Osaka, Japan) from August 2006 to September 2011. Intact human liver tissues were obtained from patients who had metastatic liver tumors or cholangiocarcinoma treated by surgical resection. The procedures for this study were in accordance with the Helsinki Declaration of 1975 (2000 revision). Liver biopsy was performed after informed consent had been granted.

Clinical Data

The age, sex, and primary clinical data for each patient were obtained on consultation or admission to our university hospital. ALT levels, albumin levels, platelet counts, and anti-HCV antibody levels were measured at the Central Clinical Laboratory of Osaka City University Medical School Hospital (Table 1).

Table 1 Characteristics of the HCV-infected patients enrolled in this study

Stage	Age[a] (years)	ALT[b] (IU/l)	Albumin[a] (g/dl)	Platelet[a] ($\times 10^3$/mm^3)	Grade (A0/A1/A2/A3)
F1	55.5 ± 13.7	63.5 (25.0–89.0)	4.1 ± 0.3	17.9 ± 5.4	3/7/0/0
F2	55.6 ± 8.7	60.2 (40.5–74.0)	3.9 ± 0.3	15.0 ± 5.4	0/5/5/0
F3	63.8 ± 8.1	75.1 (48.2–103.5)	3.8 ± 0.3	12.6 ± 3.2	0/2/5/3
F4	63.0 ± 6.8	82.4 (44.7–115.2)	3.6 ± 0.3	12.0 ± 4.1	0/4/6/0

ALT, alanine aminotransferase.
The stage of liver fibrosis and grade of necroinflammation were assessed based on the new Inuyama classification.[19]
[a]Mean ± s.d.
[b]Median (interquartile range).

Histopathological Diagnosis

Liver biopsy was performed in all 41 patients using a 15-gauge Tru-Cut needle (Hakko, Tokyo, Japan) under ultrasound guidance. The tissue samples fulfilled the size requirements suggested by Janiec *et al*:[18] adequate liver biopsy samples were defined as having a length > 1.0 cm and/or the presence of at least 10 portal tracts. The liver tissues were fixed in 10% formaldehyde, embedded in paraffin, and cut into 4-μm-thick sections. Deparaffinized sections were stained with hematoxylin–eosin and Azan–Mallory, dehydrated in 100% ethanol, cleared by xylene, mounted with NEW M·X (Matsunami Glass Industries, Osaka, Japan), and then examined by microscopy. The degree of liver fibrosis was assessed based on the new Inuyama classification[19] as follows: F0, no fibrosis; F1, expansion of the portal tracts without linkage; F2, portal expansion with portal-to-portal linkage; F3, extensive portal-to-portal and focal portal-to-central linkage; and F4, cirrhosis (Table 1).

The sections were also stained with 0.1% (w/v) Sirius red (Direct Red 80; Aldrich, Milwaukee, WI, USA) in a saturated aqueous picric acid solution for 1 h at room temperature to visualize collagen fibers. After staining, the sections were washed in two changes of 0.01 N HCl and mounted as described above.[20]

Immunostaining of Human Liver Tissues

For immunohistochemistry, paraffin sections were dewaxed in xylene and rehydrated in decreasing concentrations of ethanol (xylene: 3 × 3 min; 100% ethanol: 2 × 3 min; 95% ethanol: 3 min; 70% ethanol: 3 min). Primary antibodies and immunohistochemistry conditions are listed in Table 2. In brief, the sections were deparaffinized and treated with a solution of 3% H_2O_2 in 100% methanol for 10 min at room temperature to block endogenous peroxidase activity.

Table 2 Primary antibodies used in this study

Antibody	Species	Source	Dilution
Anti-human cytoglobin	Rb poly	Our laboratory	1/100
Anti-human cytoglobin	Mo mono	Our laboratory	1/1000
Anti-rat cytoglobin	Rb poly	Our laboratory	1/100
Anti-human cellular retinol-binding protein-1	Rb poly	Santa Cruz	1/100
Anti-human α-smooth muscle actin	Mo mono	Dako	1/100
Anti-human thymocyte differentiation antigen 1 (Thy-1)	Rb poly	Abcam	1/100
Anti-mouse thymocyte differentiation antigen 1 (Thy-1)	Mo mono	Abcam	1/100
Anti-human fibulin-2 (FBLN2)	Rb poly	Sigma	1/200
Anti-human lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1)	Rb poly	Abcam	1/200

Mo mono, mouse monoclonal antibody; Rb poly, rabbit polyclonal antibody.

The sections were then preincubated with serum-free protein block (Dako, Glostrup, Denmark) for 10 min at room temperature and subsequently incubated with primary antibodies in a dilution of 1:100 for 1 h at room temperature. Negative controls with no primary antibody were used to assess nonspecific staining. The secondary antibodies used included horseradish peroxidase-conjugated goat anti-rabbit IgG (1:200; Dako), rabbit anti-goat IgG (1:200; Dako), and rabbit anti-mouse IgG (1:200; Dako). The chromogen used was 3,3′-diaminobenzidine (Dako). The resultant sections were stained and analyzed using a BZ-8000 microscope (Keyence, Osaka, Japan).

Subsequently, double immunofluorescence staining was performed. After the paraffin sections were dewaxed, the sections were incubated with a mixture of antibodies against CYGB and α-SMA as described previously.[21] After rinsing in PBS, the sections were incubated with a mixture of fluorochrome-conjugated secondary antibodies: AlexaFluor 488 goat anti-rabbit IgG (Molecular Probes, Eugene, OR, USA) and AlexaFluor 594 goat anti-mouse IgG (Molecular Probes). The sections were briefly washed and mounted with ProLong Gold Antifade Reagent (Molecular Probes). The resulting sections were stained and analyzed using a BZ-8000 microscope (Keyence).

Morphometry for Hepatic Fibrosis

For morphometric image analysis of hepatic fibrosis in immunostaining, the areas of the liver sections that were positive for Sirius red (red), α-SMA (brown), or Thy-1 (brown) were captured separately using a charge-coupled device (CCD) camera connected to a macro digital filing system (DP70 · BX-51; Olympus Corporation, Tokyo, Japan). Images representing the whole biopsy section were acquired at × 200 magnification and digitalized. These separately captured and digitalized images were consolidated to create one large image using e-Tiling (Mitani Corporation, Tokyo, Japan). Collagen- or α-SMA-positive areas were measured using Lumina Vision 2.4 (Mitani Corporation) and were calculated automatically. The hepatic fibrotic area (%) was calculated as the area stained with the selected color divided by the whole tissue area at × 100 magnification.[20]

In fibrotic livers, the number of CYGB- and CRBP-1-positive stellate cells in each field was counted around fibrotic portal tracts (F1 to F4 samples). The analysis was performed on each 10-sample group of F1–F4 tissues using an average of five fields per zone (1.4 mm^2) (100 × objective). We counted the cell bodies that stained positively and contained a nucleus.

Cell Lines

The human HSC line LX-2 was donated by Dr Scott L Friedman at the Mount Sinai School of Medicine (New York, NY, USA).[22] LX-2 cells were cultured on plastic dishes or glass chamber slides in Dulbecco's modified Eagle's medium (DMEM; Sigma Chemical, St Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin. Huh7 cells (JCRB0403), which were obtained from the Japanese Collection of Research Bioresources (JCRB) Cell Bank (Osaka, Japan), were maintained on plastic culture plates in DMEM supplemented with 10% FBS. In some experiments, LX-2 cells were transfected with the pEGFP-cytoglobin vector (Clontech, Mountain View, CA, USA) using FuGENE HD (Roche, Applied Science, Indianapolis, IN, USA). The cells were collected at 24 h after transfection.

Preparation of Primary Cultured Mouse Hepatic Stellate Cells

Primary mouse stellate cells were isolated from 12- to 16-week-old male C57BL/6N mice (Japan SLC, Shizuoka, Japan) by pronase–collagenase digestion and subsequent purification with a single-step Nycodenz gradient, as previously described.[23] All animals received humane care. The experimental protocol was approved by the Committee

of Laboratory Animals, Osaka City University Medical School, and was performed according to institutional guidelines. Isolated stellate cells were cultured on plastic dishes or glass chamber slides in DMEM (Sigma Chemical) supplemented with 10% FBS (Invitrogen), 100 U/ml penicillin, and 100 μg/ml streptomycin. The purity of the cultures was determined based on observation of the characteristic stellate cell shape using phase-contrast microscopy.

Quantitative Real-Time PCR

Total RNA was extracted from stellate cells using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). cDNA was synthesized as previously described.[24] Gene expression was measured by quantitative real-time PCR using cDNA, THUNDERBIRD SYBR qPCR Mix Reagents (Toyobo, Osaka, Japan), and a set of gene-specific oligonucleotide primers. The reactions were performed in an Applied Biosystems Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was also measured as an internal control.

Immunoblotting

Protein samples (30 μg) were subjected to 5–20% gradient SDS-polyacrylamide gel electrophoresis (ATTO, Tokyo, Japan) and transferred to Immobilon P membranes (Millipore Corporation, Bedford, MA, USA). After blocking, the membranes were probed with a primary antibody against CYGB (1:1000; our laboratory), α-SMA (1:2000; Dako), Thy-1 (1:1000; Abcam, Cambridge, UK), or GAPDH (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The membranes were then labeled with horseradish peroxidase-conjugated secondary antibodies. Immunoreactive bands were visualized using the ECL detection reagent (GE Healthcare, Buckinghamshire, UK) and documented with an LAS 1000 device (Fuji Photo Film, Kanagawa, Japan).

Data Analysis

The data are presented as bar graphs representing the mean ± s.d. in all experiments. Statistical analyses were performed using Student's *t*-test. $P<0.05$ indicated statistical significance.

RESULTS

Specificity of the Anti-CYGB Antibody

We previously generated rabbit polyclonal anti-rat CYGB antibodies that stain stellate cells in intact and fibrotic rat liver and other visceral organs.[1,25] In the present study, we newly generated rabbit polyclonal and mouse monoclonal antibodies against human CYGB in our laboratory. Immunoblot analysis revealed that the rabbit polyclonal antibodies detected purified recombinant human CYGB,[26] which was provided by RIKEN (Harima, Hyogo, Japan), at 21 kDa and EGFP-binding recombinant human CYGB (generated in our laboratory) at 48 kDa; however, the antibodies did not detect human albumin. LX-2 and Huh 7 cells expressed negligible levels of CYGB (Figure 1a). The monoclonal antibody produced almost identical results (data not shown).

Immunohistochemical Characterization of Intact Human Liver

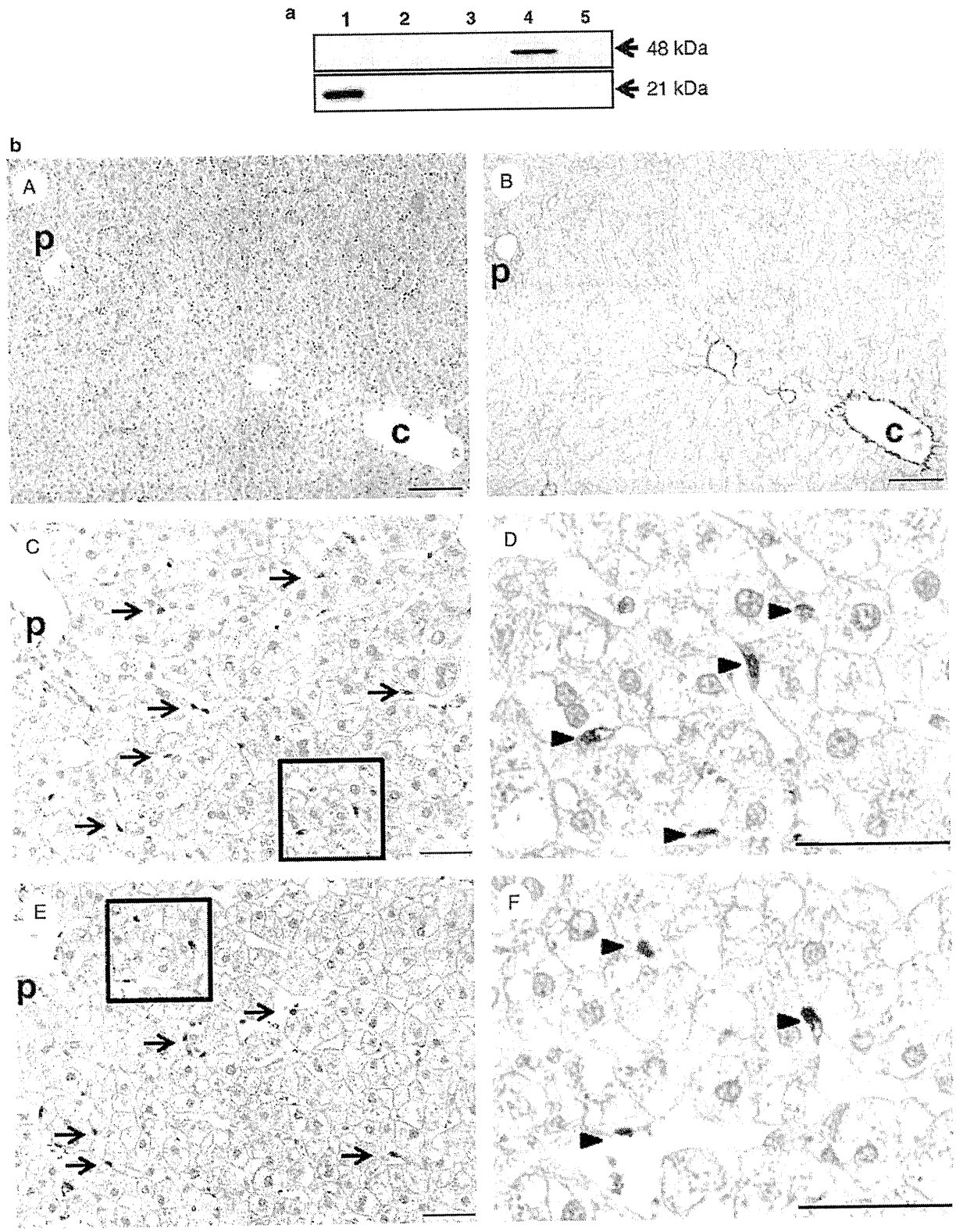
Intact human liver tissues were obtained by surgical resection from patients with metastatic liver tumors or cholangiocarcinoma, and the expression levels of CYGB, α-SMA, Thy-1, and FBLN2 were determined (Figure 1). As shown in Figure 1bA and B, the obtained tissue samples showed negligible inflammatory cell infiltration and negligible expansion of fibrotic areas. Immunostaining using the polyclonal antibodies against human CYGB revealed positive cells along the sinusoids throughout the lobule (Figure 1bC and D). Similar results were obtained with the monoclonal antibody (Figure 1bE and F). The hepatocytes and the cells in the portal areas were CYGB negative. CYGB-positive cells were present in the serial sections, and we identified these cells as stellate cells because an enlarged view revealed that they were located between the hepatocytes and the lumen of the sinusoids. In addition, these cells contained lipid droplets in their cytoplasm, and their cytoplasmic processes expanded along the sinusoids (Figure 1bD and F).

Immunohistochemistry was further performed on intact human liver samples using antibodies against CRBP-1, α-SMA, FBLN2, and Thy-1. In the liver parenchyma, strong expression of CRBP-1 was observed along the sinusoids (Figure 2a). An enlarged view showed that CRBP-1-positive cells contained lipid droplets, indicating that they were hepatic stellate cells. There were no CRBP-1-positive cells around the portal area. Instead, α-SMA-positive cells predominated; these cells also existed in the walls of the vessels, but not along the hepatic sinusoids (Figure 2b). In addition, the regions adjacent to the portal vein contained limited numbers of cells that stained for FBLN2 or Thy-1 (Figure 2c and d), which are also biomarkers of myofibroblasts.

Taken together, these findings indicate that CYGB and CRBP-1 are uniquely expressed in hepatic stellate cells in the intact human liver, whereas myofibroblast markers such as α-SMA, FBLN2, and Thy-1 are locally present in cells around the portal tract.

Immunohistochemistry of CD68, LYVE-1, and CRBP-1 in Relation to CYGB

CD68 is a glycoprotein that binds to low-density lipoprotein and is expressed by monocytes and macrophages. Kupffer cells are positive for CD68.[27] We found that spindle-shaped CD68-positive cells were present in the sinusoids in the intact human liver, indicating that these cells were Kupffer cells. These cells were predominantly located along the sinusoids (Figure 3a). Double immunofluorescence staining


a
1
2
3
4
5
48 kDa
21 kDa
b
A
p
c
B
p
c
C
p
D
E
p
F

showed that CD68 did not colocalize with CYGB (Figure 3b–d). LYVE-1, also known as extracellular link domain-containing 1 (XLKD1), acts as a receptor for both soluble and immobilized hyaluronan. Sinusoidal endothelial cells are LYVE-1 positive.[28] We observed LYVE-1 positivity along the hepatic sinusoid in the sinusoidal walls, indicating that

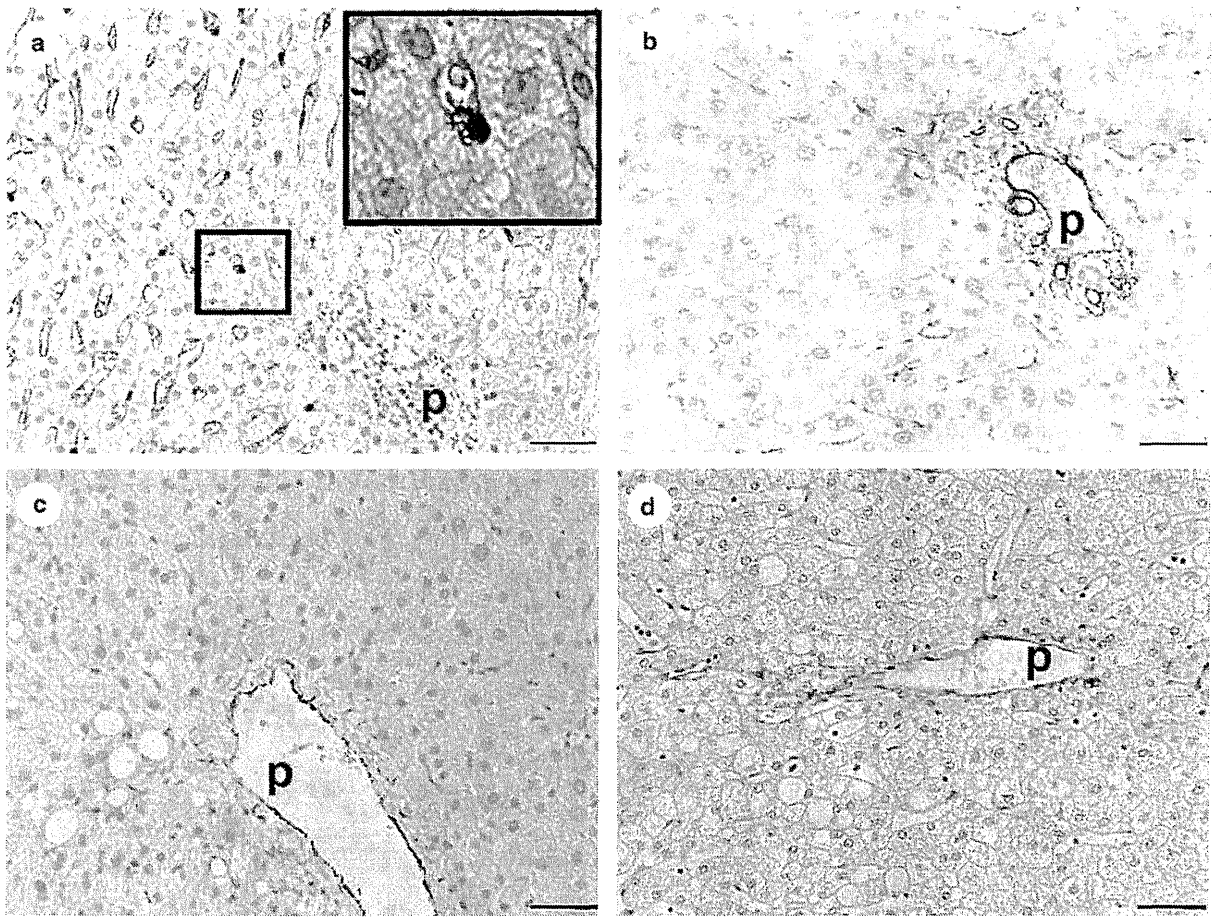


Figure 2 Immunohistochemistry for CRBP-1, α-SMA, FBLN2, and Thy-1 in intact human liver. (**a**) Immunohistochemistry for CRBP-1. In the liver parenchyma, strong expression of CRBP-1 was observed along the sinusoids (arrows). A magnified view of the enclosed area shows that a CRBP-1-positive cell contained lipid droplets, indicating that it was a hepatic stellate cell. CRBP-1 was not observed around the portal area. Bar, 100 μm. p, portal vein. (**b**) Immunohistochemistry for α-SMA. α-SMA-positive cells were present predominantly in the portal area and in the walls of the vessels, but not along the hepatic sinusoids. Bar, 100 μm. (**c**) Immunohistochemistry for FBLN2. FBLN2 was localized in the vessels of the portal spaces. Positive staining for FBLN2 was not visible along the sinusoids. Bar, 100 μm. (**d**) Immunohistochemistry for Thy-1. Thy-1-positive cells were located within the portal tracts adjacent to the wall of the portal vein. Bar, 100 μm.

Figure 1 Characterization of newly generated rabbit polyclonal antibodies against human CYGB. (**a**) Newly generated rabbit polyclonal antibodies against human CYGB detected purified recombinant human CYGB (21 kDa) (lane 1) and EGFP-labeled recombinant human Cygb (48 kDa) (lane 4) but not human albumin (lane 2). LX-2 cells and Huh 7 cells did not express CYGB (lanes 3 and 5, respectively). (1) Recombinant human CYGB (10 μg); (2) human albumin (20 μg); (3) lysate of LX-2 cells (20 μg); (4) lysate of LX-2 cells overexpressing EGFP-CYGB (20 μg); (5) lysate of Huh-7 cells (20 μg). (**b**) Immunohistochemistry of normal human liver tissues. (A) Hematoxylin–eosin staining. No hepatocyte death or inflammation was observed. Bar, 100 μm. c, central vein; p, portal vein. (B) Sirius red staining. Limited collagen deposition was observed around the portal and central vein areas. No fibrosis occurred in the liver parenchyma. Bar, 100 μm. (C) Immunohistochemistry for CYGB using newly generated polyclonal antibodies. CYGB-positive cells were localized in the liver parenchyma along hepatic sinusoids (arrows). Bar, 100 μm. (D) Magnified view of the enclosed area in (C). CYGB-positive cells were present in the perisinusoidal space and contained lipid droplets in the cytoplasm, suggesting that they were stellate cells (arrowheads). Bar, 20 μm. (E) Immunohistochemistry for CYGB using the monoclonal antibody. CYGB-positive cells were localized in the liver parenchyma along the hepatic sinusoids (arrows). Bar, 100 μm. (F) Magnified view of the enclosed area in (E). CYGB-positive cells were present in the perisinusoidal space and contained lipid droplets in the cytoplasm, suggesting that they were stellate cells (arrowheads). Bar, 20 μm.