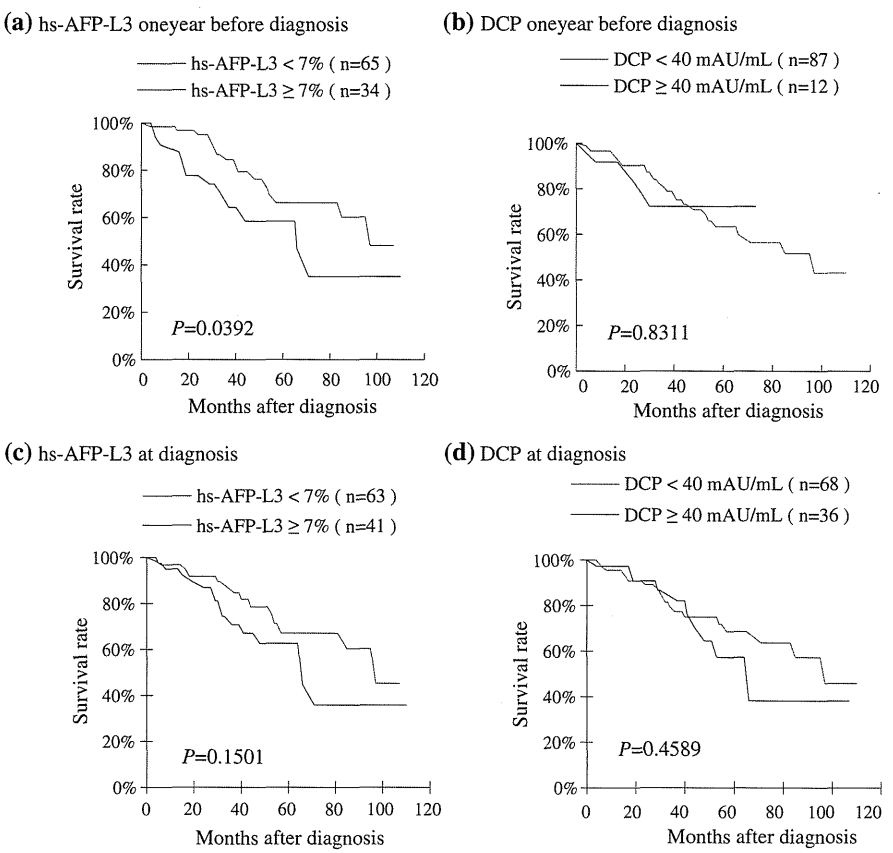

Fig. 2 Survival rates by levels of biomarkers: **a** hs-AFP-L3 and **b** DCP 1 year before, **c** hs-AFP-L3 and **d** DCP at diagnosis

patients were classified into three groups by a trigger to perform MRI for diagnostic confirmation (Table 5). US findings triggered MRI for 86 patients. The 86 patients were classified further by US findings: increase of the tumor number (51/86), increase of the tumor size (18/86), or change of the echo pattern in nodules (17/86). Five patients were monitored by MRI as results of elevated biomarkers. The remaining 13 patients were screened by MRI instead of US because interpretation of US was difficult in patients who were obese or had severe liver atrophy.

In the present retrospective study for hs-AFP-L3, 29.6 % of patients who were diagnosed with HCC by the trigger of US had hs-AFP-L3 ≥ 7 % 1 year prior to the diagnosis day. In the patients who had changes of the echo pattern in nodules, the positivity rate for hs-AFP-L3 at −1 year was 50.0 % and relatively higher compared to the other groups by US.

Table 5 Triggers to perform MRI for suspicious HCC and positivity rates for hs-AFP-L3

Triggers to perform MRI	*n*	hs-AFP-L3 >7 % At −1 year (%)	hs-AFP-L3 >7 % At diagnosis (%)
(a) Ultrasound	86	29.6	36.0
Increase of the tumor number	51	27.7	39.2
Increase of the tumor size	18	16.7	11.1
Change of the echo pattern in nodules	17	50.0	52.9
(b) Biomarkers	5	80.0	60.0
(c) Others	13	46.2	53.8

Discussion

Most studies on HCC biomarkers have focused on the accuracy at the time of diagnosis and the prediction of prognosis. So far there are a few studies which have evaluated early prediction of development of HCC in patients at high risk for HCC by biomarkers.

Taketa et al. [24] have reported that AFP-L3 values elevated above the cutoff value of 15 % with an average of 4.0 ± 4.9 months before the detection of HCC by imaging techniques. Sato et al. [25] also have demonstrated that lectin-reactive AFP elevated 3–18 months before the detection. However, only samples with AFP levels higher than 30 ng/mL were measured in their study. Recent data

indicated that the elevated AFP is not typical at HCC diagnosis for patients under in surveillance in Japan. Therefore, hs-AFP-L3 is expected to be more useful at low levels of AFP. Even though there were some differences in AFP concentration among the studies, they reported that elevation of AFP-L3 prior to diagnosis was associated with development of HCC.

Shiraki et al. [26] detected the small tumor <2 cm in maximum diameter in more than half of the patients. In the study population, they demonstrated clinical utility of lectin-reactive AFP as an early indicator while low AFP was reported limiting of the early recognition of HCC. Shimauchi et al. [27] demonstrated that AFP-L3 and DCP values showed elevated in about half of the patients at 6 months before the recognition of HCC by imaging techniques. These two markers were mutually complementary. In our study, DCP was not significantly elevated 1 year prior to diagnosis.

Lok et al. [28] have reported in a retrospective study of AFP and DCP values in patients in the Hepatitis C Antiviral Long-Term Treatment against Cirrhosis Trial who had blood drawn every 3 months for 12 months prior to HCC diagnosis. They have concluded that the biomarkers are needed to complement ultrasound in the detection of early HCC but neither DCP nor AFP is optimal. For the study, early stage HCC was defined as a single tumor nodule <3 cm in diameter with no evidence of vascular invasion or metastasis, and only 61.5 % of patients presented with early stage HCC. In our study, median of tumor size was 1.9 cm and all patients with <3 cm. Tumor volume doubling time is reported to be 90–132 days [29] and it may take a half year or 1 year for a nodule to develop from <2 cm to >3 cm. Therefore, HCC patients in our study were diagnosed 1 year earlier than the patients in Lok's study. Clinically the tumor size between <2 cm and 3 cm is one of the factor for making decisions of treatments, and it has been reported that survival rate of patients with tumor size <2 cm is higher [20]. Therefore, HCC should be diagnosed at the earlier stage with tumor <2 cm in order to achieve better outcome.

It is well known that AFP-L3 concentration correlates well with AFP; however, AFP-L3 % is not correlated with AFP [24, 30]. AFP-L3 % is a marker that is independent of AFP. Therefore, we have used AFP-L3 % for analysis.

In the present study, hs-AFP-L3 was significantly elevated 1 year prior to HCC diagnosis in 34.3 % of patients at a cutoff value of 7 %. Tamura et al. [16] reported that a cutoff value of 7 % is most appropriate for discriminating HCC from benign liver disease using this assay. Therefore, patients with elevated hs-AFP-L3 value under surveillance should be followed up closely. The specificity of 80 % or less before diagnosis may actually mislead because the non-HCC patients selected by matching with the HCC patients were potentially higher risk group for HCC and would likely develop HCC later.

In previous studies, elevated AFP-L3 has been reported to be correlated to a shorter doubling time of tumor volume, increased hepatic arterial supply, and pathologic features such as infiltrative tumor growth pattern, capsule infiltration, vascular invasion, and intrahepatic metastasis [31, 32]. These findings are often difficult to diagnose by various imaging modalities in small HCCs. Such blood supply changes typically result in change of echo pattern in nodules. In this study, therefore, high positivity rates for hs-AFP-L3 at −1 year in the patients who had such changes of echo pattern may be associated with developing HCC. The survival rate of patients with hs-AFP-L3 > 7 % at −1 year was significantly poorer compared to patients with hs-AFP-L3 < 7 %. However, differences of the detected tumor size and number were not statistically significant between patients with hs-AFP-L3 ≥ 7 % and <7 %. AFP-L3-positive HCC nodules may be aggressive and have high malignancy potential even though the tumor size is small. Therefore, it may be useful in early detection of the aggressive tumor to perform enhanced imaging techniques such as MRI for patients with elevated hs-AFP-L3. Survival rate of patients with the hs-AFP-L3 elevation at HCC diagnosis showed a poorer tendency; however, there were no statistical differences. HCC treatments were done just after the HCC diagnosis. Therefore, HCC tumors in patients with the hs-AFP-L3 elevation 1 year before HCC diagnosis might have 1 year to grow. This 1 year may reflect the difference of survival of two groups. DCP is a good marker for poor prognosis of HCC. However, the difference of overall survival between patients with DCP ≥40 and <40 mAU/mL was not observed due to the early stage (small) HCC without obvious vascular invasion.

AFP is a good marker to distinguish high-risk group for HCC development in the future [22]; however, AFP was not elevated 1 year prior to HCC development. AFP-L3 was elevated 1 year prior to diagnosis of small HCC in 34.3 % of patients.

Interpretation of US can be challenging without comparison to previous imaging results and performance of US can be limited in patients who are obese or have severe background liver cirrhosis. In the present study, sensitivity of the combined three biomarkers was 60.6 % at diagnosis, and measurements of biomarkers are expected to complement to US in surveillance.

In conclusion, elevation of hs-AFP-L3 was early predictive of development of HCC even at low AFP levels and in absence of US findings of suspicious HCC. Prognosis of patients with elevated hs-AFP-L3 was significantly poorer. HCC may be diagnosed earlier to receive curative treatments by the elevated hs-AFP-L3 as a trigger of enhanced imaging techniques. Additional prospective studies are

expected to demonstrate whether routine measurements of hs-AFP-L3 in HCC surveillance can improve overall patient survival.

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Conflict of interest All authors declare that the authors report no conflicts of interest.

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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

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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 ($\times 10^3$/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)	0.0408	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 transactivating 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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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (9): Hepatitis B virus

Role of hepatitis B virus DNA integration in human hepatocarcinogenesis

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Abstract

Liver cancer ranks sixth in cancer incidence, and is the third leading cause of cancer-related deaths worldwide. Hepatocellular carcinoma (HCC) is the most common type of liver cancer, which arises from hepatocytes and accounts for approximately 70%-85% of cases. Hepatitis B virus (HBV) frequently causes liver inflammation, hepatic damage and subsequent cirrhosis. Integrated viral DNA is found in 85%-90% of HBV-related HCCs. Its presence in tumors from non-cirrhotic livers of children or young adults further supports the role of viral DNA integration in hepatocarcinogenesis. Integration of subgenomic HBV DNA fragments into different locations within the host DNA is a significant feature of chronic HBV infection. Integration has two potential consequences: (1) the host genome becomes altered ("*cis*" effect); and (2) the HBV genome becomes altered ("*trans*" effect). The *cis* effect includes insertional mutagenesis, which can potentially disrupt host gene function or alter host gene regulation. Tumor progression is frequently associated with rearrangement and partial gain or loss of both viral and host sequences. However, the role of integrated HBV DNA in hepatocarcinogenesis remains controversial. Modern technology has provided a new paradigm to further our understanding of disease mechanisms. This review summarizes the role of HBV DNA integration in human carcinogenesis.

Key words: Hepatitis B virus; Integration; Hepatocarcinogenesis; *Cis* effect; *Trans* effect; Whole genome sequencing

Core tip: A high viral load is associated with an elevated risk of hepatocellular carcinoma (HCC), and the risk remains increased in hepatitis B surface antigen-negative hepatitis B virus (HBV) and occult infections. The ability of HBV to integrate into the infected host's hepatocyte genome is one of the most important direct pro-oncogenic properties. The recent development of efficient tools for genome-wide analysis of gene expression and genetic defects has allowed a comprehensive overview of the changes occurring with HCC. Specific HBV features, including the integration of viral DNA into host chromosomes, may trigger increased genetic instability.

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INTRODUCTION

Approximately two billion people worldwide have been infected with hepatitis B virus (HBV). With more than 350 million chronic HBV carriers, this virus is one of the most common human pathogens and is a significant public health issue[1].

Liver cancer is the sixth most common cancer, and the third leading cause of cancer-related deaths[2,3]. Hepa-

tocellular carcinoma (HCC) is the most common type of liver cancer, accounting for approximately 70%-85% of cases[4]. In recent studies conducted in Asia and Northern America, the estimated risk of developing HCC was observed to increase by 25-37-fold in hepatitis B surface antigen (HBsAg) carriers compared with non-infected patients[5,6]. HBV frequently causes liver inflammation, hepatic damage and subsequent cirrhosis. The development of liver cirrhosis is recognized as a major step in HCC pathogenesis because it occurs in 80%-90% of HCCs[7]. A high viral load is associated with an elevated risk of HCC[8], and the risk remains higher in HBsAg-negative HBV and occult infections[9-11]. HBV replication has unique characteristics[1]. HBV is classified as a pararetrovirus because of its similarity to retroviruses. In fact, HBV replicates through reverse transcription of pregenomic RNA that is an intermediate replicative molecule[12]. The ability of HBV to integrate into the infected host's hepatocyte genome is one of the most important aspects of its direct pro-oncogenic properties[13-15]. Unlike retroviruses, genomic integration has no role in HBV replication and does not produce integrase enzymatic activity protein, meaning that the integrative process is likely mediated by cellular topoisomerase I activity[16].

Integrated viral DNA is found in 85%-90% of HBV-related HCCs and its presence in tumors from non-cirrhotic livers of children or young adults further supports the role of viral DNA integration in hepatocarcinogenesis[17,18]. A significant feature of chronic HBV infection is that HBV DNA fragments are integrated into different locations within the host DNA[19-23]. Tumor progression is often associated with rearrangement and partial gain or loss of both viral and cellular sequences[24-26]. Various small-scale isolated studies have suggested that HBV integration into the host genome is a random event[25]; however, integration has been observed at chromosomal fragile sites, scaffold/matrix attachment regions, and repeat/satellite sequence-rich regions[19]. Therefore, the role of integrated HBV DNA in hepatocarcinogenesis remains controversial. This review summarizes the role of HBV DNA integration in human carcinogenesis.

HCC MECHANISMS

The are three major molecular mechanisms of hepatocarcinogenesis caused by HBV infection[27]. First, the expression of viral proteins, particularly hepatitis B virus X protein (HBx), promotes cell proliferation and viability. Second, the integration of HBV DNA into the host genome alters the expression and function of endogenous genes and induces chromosomal instability. Finally, genetic damage accumulates as a result of inflammation and ongoing hepatocyte division to replace cells killed by virus-specific T cells.

Genetic alteration plays a crucial role in cancer initiation and progression. The recent development of efficient tools for genome-wide analysis of gene expression and genetic defects has allowed a comprehensive overview of the changes occurring with HCC[28,29]. Specific HBV features, including HBV DNA integration into host genome, may trigger increased genetic instability.

Table 1 Main integration sites in human genome and in hepatitis B virus DNA

Integration sites in host genome	HBV DNA
hTERT	3′ end of HBx
MLL	Pre-S2/S
RAR-b	
CCNE1	
Cyclin A2	
FN1	
ROCK1	
SENP5	
ANGPT1	
PDGF receptor	
Calcium signaling-related genes	
Ribosomal protein genes	
Epidermal growth factor receptor	
Mevalonate kinase	
Carboxypeptidase	
Platelet growth factor receptor	

HBV: Hepatitis B virus; HBx: Hepatitis B virus X protein.

ROLE OF HBV DNA INTEGRATION IN HUMAN HEPATOCARCINOGENESIS

The association between HBV DNA integration into the host genome and HCCs was first reported in the early 1980s[13,23,30]. Subsequently, many studies were performed to further investigate this association (Table 1).

The integration of HBV DNA into host cellular DNA during HBV chronic infection disrupts or promotes cellular gene expression that is important for cellular growth and differentiation. Furthermore, the expression of HBV proteins may have a direct effect on cellular functions, and may promote malignant transformation. Integration events are thought to precede tumor development because they are found in chronic hepatitis patients and during the acute infection stage[31].

Technological limitations of PCR and Southern blot-based methods restricted previous studies that attempted to characterize the most common HBV integrant(s) in a small number of patients[15,32]. HBV has a large number of mutations at both the nucleotide and structural levels, and the lack of prior knowledge of HBV sequences in each sample may lead to PCR failure and false-negative results. This occurs when the primers are designed for deleted or polymorphic sites on the HBV genome. Recently, two studies reported "short-read" whole genome DNA paired-end sequencing of four and eighty-eight HCC patients[33,34]. Integration sites could only be inferred from paired-end reads containing both human and viral sequences, because of the limitations of the short reads generated using these platforms. Indirect roles have been proposed because the lack of identification of a dominant oncogene encoded by HBV, including insertional

activation of cancer-related genes from HBV integration, induction of genetic instability by viral integration or HBx, and long-term effects of viral proteins that enhance immune-mediated liver disease.

Integration has two potential consequences: (1) the host genome becomes altered ("*cis*" effect); and (2) the HBV genome becomes altered ("*trans*" effect). The cis effect includes insertional mutagenesis, which can potentially disrupt host gene function or alter host gene regulation [*e.g.*, telomerase reverse transcriptase (TERT)][35]. Despite drastic rearrangements, the coding regions of PreS2 and HBx were generally conserved and could be transcribed[36]. Hence, these two HBV proteins may have a *trans* role in hepatocarcinogenesis[37-39].

CIS EFFECT

The main integration sites in the human genome and the preferred integrating region within the HBV genome have been researched extensively.

HBV DNA integration occurs randomly within human genomes, and may involve multiple sites in different chromosomes[25]. Thus, HBV behaves like an insertional, non-selective mutagenic agent. The important host genome rearrangements associated with viral integration suggest that the main oncogenic effect is from the induction of higher genomic instability[40]. Most reported integration events occur near or within fragile sites or other repetitive regions, such as the Alu sequences and microsatellites that are prone to instability, tumor development, and progression[22]. Integration of HBV DNA sequences begins in the early stages of acute infections, and multiple integrations have been detected in chronic hepatitis tissues. Clonal integrated HBV sequences have been observed in approximately 80% of HBV-related HCCs[41]. Viral insertion sites have been mapped in multiple regions on virtually all chromosomes, suggesting a random distribution throughout the host genome. HBV insertions are commonly associated with large genetic alterations that may lead to the abrogation of control mechanisms that safeguard chromosomal integrity[42-45]. Similar to retroviral proviruses, HBV DNA targets actively transcribed chromosomal regions within genes or in the immediate vicinity. Sequence analysis of multiple viral-host junctions have identified cellular coding regions within several kbps in 90% of cases, with frequent targeting of gene families involved in cell survival, proliferation and immortalization including: hTERT, the PDGF receptor, MLL, calcium signaling-related genes and ribosomal protein genes[15]. These findings favor the view that viral insertion induces the first genetic alteration in tumor development. Target genes may play a role in hepatocarcinogenesis, which was previously shown for HBV insertions into the retinoic acid receptor b (RAR-b) and the cyclin A2 genes[46,47].

Among the numerous viral integration sites described, some notable regions include the tyrosine-protein-kinase domain of the epidermal growth factor receptor gene[48], the mevalonate kinase gene[49,50], the carboxypeptidase gene[51], platelet growth factor receptor genes[15] and *hTERT*.

The HBx gene in the HBV genome tends to be the most common region, but the most common integration sites in the human genome are not fully identified. Several integration sites in the human genome such as *TERT*, *MLL4*, *CCNE1*, *FN1*, *ROCK1* and *SENP5* have been reported[33-52]. *TERT* encodes a telomerase reverse transcriptase, which plays an essential role in overriding cellular senescence. Its dysregulation in somatic cells is linked to carcinogenesis[53]. *MLL4* encodes a histone methyltransferase that plays a critical role in gene expression and epigenetics in cancer cells. The translocation breakpoint of the intron 3 region of *MLL4* is one of the preferential targets for HBV DNA integration and may be involved in liver oncogenesis[54]. *CCNE1* encodes cyclin E1, which is required for cell cycle G1/S transition. *FN1* encodes fibronectin, a component of the extracellular matrix that is involved in cell adhesion and migration processes. The protein encoded by *ROCK1* can activate LIM kinase, and inhibits actin-depolymerizing activity by phosphorylating cofilin. *SENP5* encodes a protease specific for SUMO proteins, and is required for numerous biological processes. All of these genes are upregulated in malignant tissues[34]. Hence, HBV integration into these genes may cause HCC.

Whole genome sequencing (WGS) of a large cohort has provided an opportunity to identify novel recurrent integrations. In addition to the confirmation of recurrent HBV integration into the *MLL4* (n = 9) and *TERT* (n = 18) loci accompanied by upregulation of gene expression, recurrent integration events were observed at the *CCNE1* (n = 4), *SENP5* (n = 3), and *ROCK1* (n = 2) loci[34]. *CCNE1* expression was, on average, 30-fold higher in tumors with HBV integration compared to the normal controls. Cyclins are mainly involved in regulating the cell cycle in eukaryotic cells, and are major targets for oncogenic signals. HBV integration at the *CCNE1* locus has provided at least one molecular mechanism driving aberrant cell cycle control leading to HCC. Currently, three genome-sequencing studies have been published that analyzed HBV integration events. Genome sequencing of four HCC patients identified 255 HBV integration sites in the three HBV-positive patients including the *MLL4* locus in one sample and the *ANGPT1* locus in another[33]. RNA sequencing revealed a distinct transcriptional impact of viral integration. HBV DNA integration into the third exon of *MLL4* resulted in a human-viral fusion transcript, and a 20-fold increase in *MLL4* transcription in comparison to the adjacent normal liver tissue. For the *ANGPT1* gene, HBV DNA was inserted into 10-kb upstream of the promoter region, leading to a greater than eightfold elevation in *ANGPT1* expression. In a genome sequencing study of 27 HCCs, including 11 HBV-associated HCC, 14 HCV-associated HCC, and two cases that were unrelated to viral infection, the average proportion of the *TERT* integration sites (41%) was higher than that of other integration sites. These findings are consistent with previous reports of recurrent HBV integration at the *TERT* locus[55].

Preferential HBV integration into gene promoters ($P < 0.001$), and significant enrichment of integration into chromosome 10 ($P < 0.01$) was observed in the tumors. Integration into chromosome 10 was significantly associated with poorly differentiated tumors ($P < 0.05$). In particular, recurrent integration into the *TERT* promoter was correlated with increased TERT expression[56].

We found that HBV DNA integration enhanced host chromosomal instability leading to large inverted duplications, deletions and chromosomal translocations[32]. Many of these chromosomal segments contain genes encoding key factors in liver carcinogenesis, such as p53, Rb, Wnt/b-catenin, cyclins A and D1, TGFb, and Ras[57].

TRANS EFFECT

Integrated viral sequences may contribute "*in trans*" to tumorigenesis through the production of truncated and mutated HBx or preS2/S proteins, though they cause defective replication. These proteins may impact HCC development by disrupting cellular gene expression control or by activating oncogenic signaling pathways.

The HBx protein is a multifunctional regulator of viral and cellular genes that interferes with viral replication and proliferation. HBx and Pre-S2/S regulatory proteins that are generated from integrated viral sequences are involved in hepatocyte transformation. Moreover, HBx and truncated Pre-S2/S have been shown to be effective transactivators of cellular and viral genes and are involved in signal transduction pathways, cell cycle control and transcriptional regulation[36,58].

The C-terminal region of HBx, produced by HBx truncation, contributes to HCC development. It has been suggested that the C-terminal region is required for reactive oxygen species (ROS) production and 8-oxoguanine (8-oxoG) formation, which are biomarkers of oxidative stress. Oxidative stress and mitochondrial DNA damage play an important role in the development of HCC[59]. Other studies have found that HBx C-terminal truncation, particularly involving 24 amino acids, plays a role in enhancing cell invasiveness and metastasis in HCC by activating MMP10 through C-Jun signaling[60]. Also, HBx C-terminal truncation was closely related to the overexpression of centromere protein A in HCC[61]. In addition, HBx C-terminal truncation directly regulates miRNA transcription and promotes hepatocellular proliferation[62].

Most HBV-related HCCs have integrated viral genomic sequences, including the HBx gene. Although the integrated forms of HBx are frequently rearranged and show numerous point mutations, deletions or truncation, integrated HBx may encode functionally active proteins with transactivating ability[31,41]. Characterization of HBx expression in malignant hepatocytes and infected liver tissues has been often hampered by the difficulty in obtaining valid high-affinity anti-HBX antibodies for immunodetection[63]. Despite this, the expression of HBx is maintained through multistage hepatocarcinogenesis from pre-neoplastic nodules or foci of transformed hepatocytes to HCC[64,65].

Evidence of transcriptional activity at integrated X sequences has been demonstrated in tumors and chronically infected livers[66,67] and may be correlated with the detection of the X protein in human HCCs[68]. It was suggested that downstream cellular sequences contribute to activated expression and/or enhanced transactivating capacities of the integrated HBV sequences[58,69]. The X gene product transactivates homologous and heterologous transcriptional enhancers and promoter sequences. In the meantime, expression of cellular genes is activated *"in trans"* from increased X gene products. Many clones preserved transactivation activity in spite of the truncation at the 3' end of the X ORF[67]. The cDNA structure of X mRNA from integrated HBV DNA suggested X-cell fusion mRNA.

The preferred region within the HBV genome involved in integration and viral structural alteration is located at nucleotides 1600-1900 around the 3'-end of HBx and the 5'-end of the Precore/Core genes, where viral replication and transcription is initiated. Upon integration, the 3'-end of HBx is frequently deleted and HBx-human chimeric transcripts, which can be expressed as chimeric proteins, are commonly observed[56]. The 3'-end of the HBx gene is the preferred region for human genome integration[34,52,70], leading to the C-terminal truncated form of HBx, and is an important mechanism in HBV-related hepatocarcinogenesis.

Recently, WGS was performed on a large cohort of HCC patients with 81 HBV-positive, seven HBV-negative HCC samples and adjacent normal tissues to survey HBV integration in liver cancer genomes[34]. A systematic and in-depth bioinformatics analysis was performed to study HBV integration. The 399 detected HBV integration events occurred more frequently in tumors (344 events) than the normal controls (55 events), and represented a 6.3-fold increase. The HBV genome break points were also examined, and 40% of the break points were restricted to an 1800-bp region of the HBV genome where the viral enhancer, the X gene and the core gene are located. This viral breakpoint may facilitate the formation of human-viral fusion proteins and create cis-regulatory effects on expression of downstream genes that disturb the host gene regulatory network.

Some HCC patients do not have detectable hepatitis B surface antigen in their serum, but have low levels of serum HBV DNA and fragments of HBV DNA in their genomic cellular DNA (occult HBV infections). The prevalence and molecular status of occult HBV in HCC patients has been investigated in many studies in patients from different regions worldwide[10,71,72]. In HBsAg-negative HCC patients, HBV DNA was detected in neoplastic and/or adjacent non-neoplastic liver tissue in almost half of patients, some of which were anti-HCV positive[73]. In some patients, positivity for anti-HBc antibodies was the only marker of HBV infection. Covalently closed circular HBV DNA may be detected in the liver of some patients, indicating persistence of the viral genome template for transcription and replication. An observational cohort study showed that HCC develops more commonly in oc-

cult HBV patients among HBsAg-negative patients with chronic hepatitis C.

In addition to genetic and genomic perturbations, HBV integration is also associated with various clinical parameters including disease occurrence at younger age, higher levels of AFP and poor overall survival[34]. This suggests an association between viral DNA integration and a more aggressive pathogenesis of HCC.

Beside genomic alterations, epigenetic factors, such as methylation-associated gene silencing, have been shown to be involved in the deregulation of cellular function in HCC. The HBV genome is almost completely unmethylated in the early stages of carcinogenesis, from chronic active hepatitis to hepatic cirrhosis, while it becomes more methylated in the established liver tumors, both in patients and in cultured cancer cell lines[74].

CONCLUSION

The multistep development of liver cancer is associated with the accumulation of genetic and epigenetic changes. The long latency of HCC development following primary HBV infection reflects an indirect oncogenic pathway. Evidence of multiple cooperative mechanisms during neoplastic transformation is increasing. Genetic instability, which is particularly high in HBV-related HCCs, may be related to HBV integration.

The integration of HBV has the primary *cis* effect of altering gene regulation. Sequence variations and structural alterations of the HBV genome that modify viral protein structure, function and integration events generate novel HBx-human chimeric proteins that may exert a *trans* effect by facilitating host immune surveillance evasion and/or that contribute to tumorigenesis.

Next generation sequencing technology has provided a new paradigm for understanding disease mechanisms. WGS and whole exome sequencing efforts have led to the discovery of previously unknown somatic variations in HCC, such as point mutations in chromatin remodeling genes and recurrent HBV integrations. A large number of data sets from genome wide association studies may need further investigation. Additional research into the development and treatment of resistant HBV strains is warranted.

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