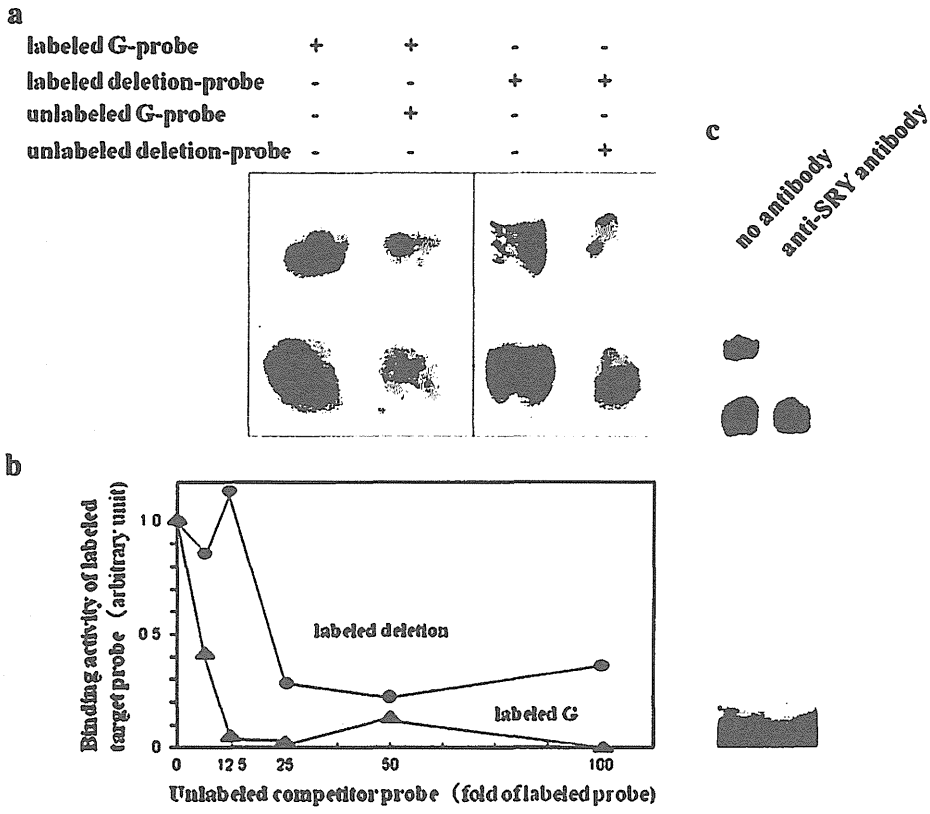

Fig. 2 Electrophoretic mobility-shift assay (ems) with oligonucleotides around nt -155. a EMS was performed with oligonucleotides carrying either deletion mutation or allele G at nt -155 and nuclear extracts from HepG2 cells, and two complex bands were found. In the specific competition binding assay, the respective unlabeled oligonucleotides were added at a fivefold molar excess. b Specific cross-competition binding assay was performed for the retarded complex band. Unlabeled oligonucleotides were added as competitors at 6.25-, 12.5-, 25-, 50-, and 100-fold molar excess. c Anti-human SRY and nuclear extracts were incubated for 30 min at 4 °C before the addition of oligonucleotides with deletion mutation at nt -155

In contrast, the nuclear protein derived from HepG2 cells produced one complex band with oligonucleotides around nt -443 (Fig. 3a), and the binding activity was slightly greater in the case of oligonucleotides with allele T than in those with allele C (Fig. 3b).

SRY expression in the HepG2 cells

The expression of SRY was confirmed in the HepG2 cells; a 27 kDa protein reacting with anti-SRY was observed by western blotting (Fig. 4a), and amplification bands of 270 and 612 bp, respectively, corresponding to the short and long cDNAs of SRY were detected by RT-PCR (Fig. 4b).

Discussion

In the present study, SNPs in the promoter region of *OPN* were evaluated in patients with chronic hepatitis C and HCC. In the present study, HCC was found in 120 of 296 patients; the incidence of HCC development was 40.5 % among those with HCV infection. The present study was done both through retrospective and prospective manners. Thus, there existed patients with HCC already at the enrollment of the study. Also, a lot of patients with advanced liver diseases were introduced to our hospital from clinics in Saitama Prefecture. These situations may produce a high percentage of HCC patients among those with HCV infection.

SNP at nt -155 was selected among the three SNPs showing linkage disequilibrium with each other, and the allele at nt -155 as well as that of the SNP at nt -443 were determined. We found that the peripheral blood platelet counts at the detection of HCC were greater in women patients with homozygous deletion at nt -155 and C/C or C/T at nt -443 than in those showing other combinations of alleles, while no such difference was seen in the men patients. The functions of these SNPs were assessed by the dual-luciferase reporter assay in the HepG2 cells. Also, the regulatory mechanisms of *OPN* transcription were analyzed through EMSA. Consequently, we revealed that the promoter activity was greater in the oligonucleotide with deletion at nt -155 and C at nt -443 than in those with other haplotypes, and that SRY and indeterminate transcriptional factors having binding capacity for either the oligonucleotide with deletion mutation at nt -155 or for that with allele T at nt -443 may contribute to the regulation of *OPN* expression in the HepG2 cells.

It is well known that the peripheral blood platelet counts decrease and the likelihood of HCC development increases with the progression of liver fibrosis in patients with persistent HCV infection [38, 39]. In the present study, the age

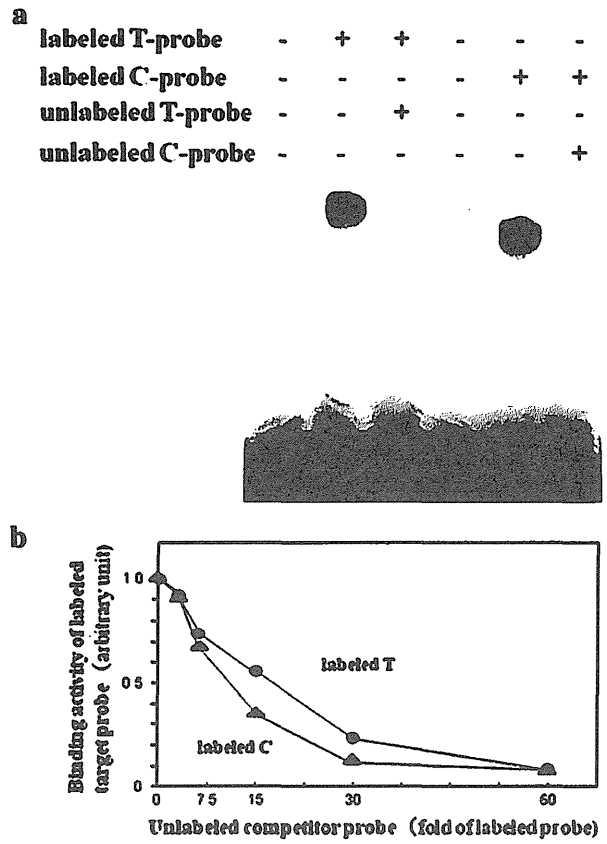

Fig. 3 Electrophoretic mobility-shift assay (EMS) with oligonucleotides around nt -443. a EMS was performed with oligonucleotides carrying either allele C or T at nt -443 and nuclear extracts from HepG2 cells, and one complex band was visualized. In the specific competition binding assay, respective unlabeled oligonucleotides were added at a fivefold molar excess. b Specific cross-competition binding assay was performed with unlabeled oligonucleotides as competitors at 3.75-, 7.5-, 15-, 30-, and 60-fold molar excess

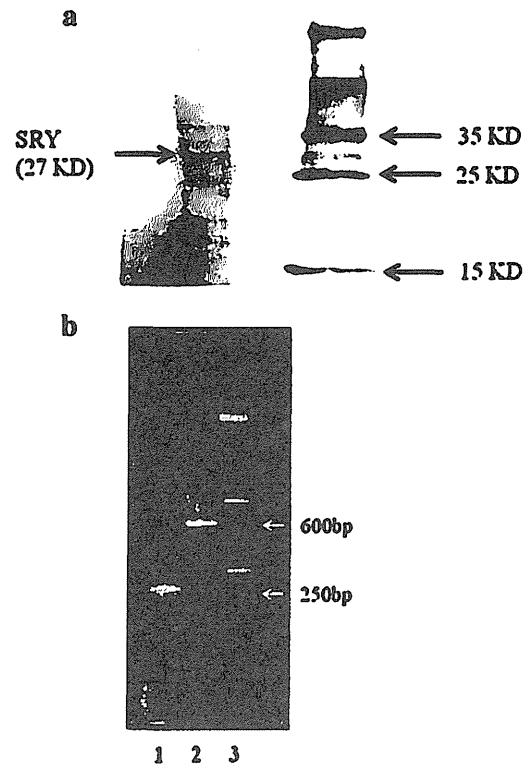

Fig. 4 SRY expression in HepG2 cells. a SRY protein expression. Western blot analysis was performed using HepG2 cell lysates and anti-human SRY, and a 27 kDa band specific for SRY was visualized. b SRY mRNA expression in HepG2 cells. RT-PCR revealed amplified products of SRY short cDNA (270 bp; *lane 1*) and SRY long cDNA (612 bp; *lane 2*)

at the detection of HCC was significantly higher in women than in men, whereas the peripheral blood platelet counts at HCC detection were lower in women than in men, suggesting that HCC occurred in the later stage of liver fibrosis in women patients than in men patients. These observations are in line with findings reported previously [24, 29]. It is noteworthy, however, that the peripheral blood platelet counts were higher in women patients with homozygous deletion at nt -155 and C/C or C/T at nt -443. Thus, HCC may develop in the early stage of liver fibrosis after HCV infection, especially in women with such a genetic background.

In the present study, plasma *OPN* concentrations were not measured both in patients with and without HCC, since the study was done retrospectively and blood samples at the detection of HCC were not stocked. Kim et al. [40] reported that plasma *OPN* levels were higher in patients with HCC than in those without HCC and the levels increased in patients with HCC in relation to the staging of HCC progression. These observations suggest that plasma *OPN* levels as well as *OPN* expression in the liver may differ among patients with HCV depending on the alleles of *OPN* promoter gene, and the differences of *OPN* expressions might influence the development of HCC in these patients. These matters should be investigated in the future through the study in a prospective manner.

According to the results of the dual-luciferase reporter assay, the hepatic expression of *OPN* may be upregulated in patients with homozygous deletion at nt -155 and C/C or C/T at nt -443, in whom HCC developed in the early stage of liver fibrosis, especially in women patients with HCV infection. Zhao et al. [15] reported that the downregulation of *OPN* in HCC cells inhibited cell growth and invasion through extracellular matrix in an autocrine manner in vitro and suppressed carcinogenicity and metastasis to the lung in nude mice. They also suggested that *OPN* may facilitate carcinogenesis through the prevention of apoptosis in HCC cells [15]. Moreover, Sun et al. [14] revealed that *OPN* was essential for the proliferation of HCC cells, but the levels required for cell growth were much lower than those

necessary for the invasion and metastasis of the cells. Considering these observations, it is possible that *OPN* is increasingly expressed in the liver of women patients with homozygous deletion at nt -155, and that C/C or C/T at nt -443 might enhance the progression of HCC even in the early stage of liver fibrosis following HCV infection.

Two bands of binding complexes were visualized by EMSA of nuclear extract proteins from HepG2 cells and oligonucleotides around nt -155 in the promoter region of *OPN*. The binding activity of the retarded complex was shown to be greater in the oligonucleotides with deletion mutation at nt -155 than in those with allele G, based on the specific cross-competition binding assay, while the other complex showed the same binding activity to oligonucleotides, irrespective of the allele. According to computer simulation, transcription factor SRY has the ability to bind to the promoter region of *OPN* around nt -155, irrespective of the alleles associated with the SNP. In the present analysis, the incubation of the nuclear extract with anti-SRY before the addition of oligonucleotides diminished the latter complex band, in which the binding activities to oligonucleotide were similar between the two alleles. It remains controversial as to whether SRY is expressed in adult cells as well as embryo cells [41–43], but we confirmed SRY expression at both the protein and mRNA levels in HepG2 cells cloned from the HCC cells of a men patient. These findings suggest that HCC progression might be accelerated in women patients, especially in those with deletion mutation at nt -155 through the upregulation of *OPN* by the increased binding activity between an indeterminate transcriptional factor and the promoter region around nt -155, since SRY is not expressed in women. In the case of men patients, the effect of the indeterminate transcriptional factor, being dependent on the allele at nt -155, might be reduced in the presence of SRY, which may promote the transcription of *OPN* to enhance HCC progression, irrespective of the allele at nt -155. To confirm our hypothesis, mRNA and protein expressions of SRY should be evaluated in HCC tissues in men patients.

Computer simulation presented that the transcription factor FoxD3 had the ability to bind to the promoter region of *OPN* around nt -155 only when the allele was deletion mutation. Giacopelli et al. [22] reported that runt-related transcription factor 2 (RUNX2) bound more tightly to oligonucleotides around nt -155 in the case of the presence of allele G than in the case of the presence of deletion mutation. However, the retarded band which showed a superior binding activity to oligonucleotides with deletion at nt -155 by EMSA, did not disappear following the addition of the anti-FoxD3 antibody or anti-RUNX2 antibody in our experiments (data not shown). On the other hand, one binding complex band was observed by EMSA with oligonucleotides around nt -443 of the promoter region of *OPN*, and the binding activity was slightly greater in vitro in the case of the oligonucleotides with allele T than in those with allele C. Computer program simulation proposed that the transcriptional factor CDxA can bind to a similar region in the case of the presence of allele T at nt -443. Schultz et al. [23] reported that the DNA-binding domain in the transcriptional factor c-Myb had the capacity to bind to the oligonucleotides around nt -443 through EMSA analyses. In the present study, a transcriptional factor expressed in HepG2 cells, which can bind to oligonucleotides around nt -443, could not be clarified. However, it is suggested that such a transcriptional factor might suppress *OPN* transcription both in men and women patients with allele T at nt -443 in the promoter region of *OPN*. These indeterminate factors, therefore, should be further clarified.

In conclusion, SNPs in the promoter region of the *OPN* gene may play a role in the sexual difference in the risk of HCC development in patients with persistent HCV infection, probably through the regulation of the transcription of *OPN* in the liver. SRY and indeterminate transcriptional factors that have binding capacity for the promoter region of *OPN* either at nt -155 or nt -443 may be involved in the transcriptional regulation.

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Classification of the Etiologies of Acute Liver Failure in Japan
- A Report by the Intractable Hepato-Biliary Diseases Study Group of Japan -

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ABSTRACT: The Intractable Liver Diseases Study Group of Japan, supported by the Ministry of Health, Labour and Welfare, established novel diagnostic criteria for "acute liver failure" in 2011. In these criteria, patients without histological findings of hepatitis are included in the disease entity of "acute liver failure," as in Europe and the United States. In this report, classification criteria for the etiologies of "acute liver failure" in Japan are proposed.

Hepatitis viral infection is the most important and common cause of acute liver failure in Japan. Acute liver failure is typically represented by fulminant hepatitis in Japan, and the diagnostic criteria for "fulminant hepatitis" were established by the Inuyama Symposium in 1981 [1]. The etiology of fulminant hepatitis includes viral infections, autoimmune hepatitis, drug allergy-induced liver injuries, and hepatitis of indeterminate etiologies [2]. In contrast, in the United States, Trey and Davidson proposed criteria for the diagnosis of "fulminant hepatic failure" in 1970 [3], which includes liver failure caused by drug toxicity, circulatory disturbances, metabolic diseases, acute fatty liver of pregnancy, and postoperative liver damage, none of which is included in the etiological factors of the disease entity of "fulminant hepatitis" in Japan. Then, Polson and Lee published an AASLD position paper in 2005 [4], and "fulminant hepatic failure" was replaced by "acute liver failure", although the etiological factors of the disease entity of "acute liver failure" have not been changed until now, either in Europe or in the United States.

The diagnostic criteria for "fulminant hepatitis" in Japan need to be revised to correspond to those for "acute liver failure" in Europe and the United States. Thus, the Intractable Liver Diseases Study Group of Japan, supported by the Ministry of Health, Labour and Welfare, established novel diagnostic criteria for "acute liver failure", which includes the disease entity of "fulminant hepatitis" in 2011 [5,6]. According to these criteria, patients showing prothrombin time values of 40% or less of the standardized values or INRs of 1.5 or more caused by severe liver damage developing within 8 weeks of the onset of symptoms are diagnosed as having "acute liver failure", with the liver function prior to the current onset of liver damage being estimated to be normal. Patients without histological findings of hepatitis are included in the disease entity of "acute liver failure," as in Europe and the United States. In this report, classification criteria for the etiologies of "acute liver failure" in Japan are proposed (*Table 1*).

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Table 1. Classification of the Etiologies of Acute Liver Failure

I. Viral infection; those satisfying the following criteria for laboratory data, showing clinical features consistent with viral infection.

I-① HAV: Positive test result for anti-HAV (IgM)

I-② HBV: Positive test result for either HBs-antigen or anti-HBc (IgM), but care should be exercised in rare cases in which the test result for serum HBV-DNA is positive whereas all of the serum markers for HBV are negative*

I-②-1. Transient HBV infection; when any of the following 3 situations is satisfied.

- Negative test result for HBs antigen preceding the onset of liver injury in the absence of immunosuppressive and/or anticancer therapies in the previous 12 months
- High levels of anti-HBc (IgM)
- Low levels of anti-HBc (IgG)

I-②-2. Acute Exacerbation in HBV carriers; when any of the following 4 situations is satisfied.

- Positive test result for HBs antigen preceding the onset of liver injury (A)
- Low levels of anti-HBc (IgM) (B)
- High levels of anti-HBc (IgG) (C)
- Negative test result for HBs antigen, but positive test results for anti-HBc or anti-HBs preceding the onset of liver injury, in cases with a history of immunosuppressive and/or anticancer therapies in the previous recent 12 months (D)

I-②-2-i. Asymptomatic or inactive HBV carriers without drug exposure; those satisfying A, B or C above in the absence of immunosuppressive and/or anticancer therapies in the previous 12 months

I-②-2-ii. Reactivation in asymptomatic or inactive HBV carriers receiving immunosuppressive and/or anti-cancer drugs; those with a history of immunosuppressive and/or anticancer therapies in the previous 12 months satisfying A, B or C above.

I-②-2-iii. Reactivation by immunosuppressant and/or anticancer drugs in patients with resolved HBV (*de novo* HBV hepatitis); those satisfying D

I-②-3. Indeterminate HBV infection; those with HBV infection, but not fulfilling the criteria shown in I-②-1 and I-②-2.

* To bear in mind that in general, hepatitis due to HBV is associated with high levels of serum HBV-DNA, except in HBe-antigen-positive asymptomatic carriers.

I-③ HCV: Positive for anti-HCV and/or HCV-RNA

I-④ HEV: Positive for anti-HEV (IgA) and/or HEV-RNA

I-⑤ Other viruses: Demonstration of transient infection or reactivation of EB virus, cytomegalovirus and other viruses through measurements of serological markers and viral genomes.

II. Autoimmune hepatitis; those satisfying "Criteria for Diagnosis of Autoimmune Hepatitis" proposed by the International Autoimmune Hepatitis Group, or those positive for antinuclear antibody or serum IgG concentrations 1.1 times the upper limit of the normal range at each institution or greater**

** To bear in mind that patients with autoimmune hepatitis might be confused with those having drug-induced liver injuries or hepatitis of indeterminate etiology. Patients with the possibility of this condition should be treated as soon as possible as cases for autoimmune hepatitis.

III. Drug-Induced Liver Injuries; those consistent with drug-induced liver injury based on their clinical courses.

III-① Drug allergy-induced hepatitis***

III-② Drug toxicity-induced liver injury (excluded from hepatitis)***

*** Differential diagnosis between drug allergy-induced hepatitis and drug toxicity-induced liver injuries is based on the types and doses of the drugs and the clinical features of the patients.

IV. Liver injuries without the histological findings of hepatitis; diagnosis is based on the clinical features of the patients.

IV-① Circulatory disturbance****

IV-② Metabolic diseases; Wilson's disease, anorexia nervosa, acute fatty liver of pregnancy, Reye's syndrome and others.

IV-③ Infiltration of the liver by malignant cells

IV-④ Liver injuries after liver resection and transplantation

IV-⑥ Miscellaneous etiologies

**** Liver injuries after operation other than liver resection and transplantation, those due to bacterial infection, DIC and heat stroke are in general classified as being caused by circulatory disturbance

V. Indeterminate etiology despite adequate examinations

VI. Unclassified due to inadequate examinations

Correlation Between Hepatitis B Virus Surface Antigen Level and Alpha-Fetoprotein in Patients Free of Hepatocellular Carcinoma or Severe Hepatitis

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Alfa-fetoprotein (AFP) is used as a marker of early hepatocarcinogenesis. However, the impact of hepatitis B virus surface antigen (HBsAg) on this relationship in patients with HBV infection is not clear. The present study evaluated the relation between HBsAg and AFP levels at the initial visit in 1,610 untreated HBV patients, free of hepatocellular carcinoma (HCC) or severe hepatitis. The cumulative rate of HCC was significantly lower in patients with a low AFP level (≤10 μg/L; below the upper limit of normal) than in those with a high AFP level (≥11 μg/L) at the initial visit. In patients with HBsAg levels more than 500 IU/ml, HBsAg levels correlated significantly and negatively with AFP levels, and significantly with platelet count. Multivariate analysis of data of patients with HBsAg more than 500 IU/ml identified HBsAg (<7,000 IU/ml), albumin (<3.9 g/dl), platelet count ($<20.0 \times 10^4/mm^3$), gamma-glutamyl transpeptidase (≥50 IU/L), aspartate aminotransferase (≥34 IU/L), HBeAg (positive), and HBV core-related antigen (≥3.0 log U/ml) as determinants of a high AFP. Especially, in patients with HBsAg more than 500 IU/ml and low transaminase levels (below the upper limit of normal), HBsAg was identified as significant determinant of a high AFP. On the other hand, in patients with HBsAg less than 500 IU/ml, multivariate analysis identified albumin, gamma-glutamyl transpeptidase, and HBV core-related antigen as determinants of a high AFP. The results indicated that HBsAg level seems to affect, at least in part, the AFP levels, and that it can be used as a surrogate marker of early hepatocarcinogenesis. ***J. Med. Virol. 86:131–138, 2014.*** © 2013 Wiley Periodicals, Inc.

KEY WORDS: HBV; AFP; HBsAg; HBcrAg; genotype; hepatocellular carcinoma

INTRODUCTION

Hepatitis B virus (HBV) is a small, enveloped DNA virus known to cause chronic hepatitis and often leads to liver cirrhosis and hepatocellular carcinoma (HCC) [Viola et al., 1981; Kobayashi et al., 2002; Yao, 2003]. Evidence suggests that the use of elevated alpha-fetoprotein (AFP) for the prediction of early hepatocarcinogenesis in non-HCC patients could be clinically useful. AFP is a fetal glycoprotein produced by the yolk sac and fetal liver [Bergstrand and Czar, 1956] and has been widely used as a serum marker for the diagnosis of HCC [Sato et al., 1993; Johnson, 2001]. Furthermore, high serum AFP levels are also associated with various chronic liver diseases and hepatic regeneration [Kew et al., 1973; Silver et al., 1974; Elftherious et al., 1977; Alpert and Feller, 1978]. Many patients with chronic hepatitis B who are free of HCC have high AFP levels [Chen and Sung, 1979; Di Bisceglie and Hoofnagle, 1989], and some patients with cirrhosis and concomitant high

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inflammatory activity have very high AFP levels [Yao, 2003; Cheema et al., 2004]. On the other hand, some patients with small HCC lesions have only moderately elevated levels of AFP [Shinagawa et al., 1984; Ebara et al., 1986; Bruix and Sherman, 2005]. At present, however, there are no cutoff levels for serum AFP used to predict HCC in patients with HBV infection.

There is growing interest in the use of hepatitis B surface antigen (HBsAg) level as a prognostic marker in chronic hepatitis B patients [Chan et al., 2010]. The HBsAg levels are useful for identifying the stage of disease [Jaroszewicz et al., 2010; Nguyen et al., 2010], to distinguish true inactive carriers from patients with HBe antigen-negative disease [Brunetto et al., 2010; Martinot-Peignoux et al., 2010; Chan et al., 2011; Liaw, 2011], and to predict the response to interferon therapy [Brunetto et al., 2009; Moucari et al., 2009]. Recent studies has also demonstrated that the HBsAg levels are associated with the risk of progression to HCC, especially in patients with low HBV DNA levels [Chan, 2012; Tseng et al., 2012], and that there is a potential correlation between the HBsAg levels and the stage of liver fibrosis [Seto et al., 2012; Martinot-Peignoux et al., 2013]. However, the impact of viral factors, such as the HBsAg level, on serum AFP level as a predictor of early HCC is not clear at present.

The present study included 1,610 untreated patients with HBV infection, free of HCC or severe hepatitis. The present study was designed to provide answers to the following questions: (1) what is the relation between a high serum AFP level at the initial outpatient visit and subsequent development of hepatocarcinogenesis in antiviral-therapy-naive patients with hepatitis B viral infection? (2) What is the impact of viral factors, such as the HBsAg level, on serum AFP level in such patients, and (3) What is a good surrogate marker for a high serum AFP at the initial visit.

PATIENTS AND METHODS

Patients

Among 6,466 consecutive patients who were diagnosed with HBV infection between March 1972 and December 2012 at Toranomon Hospital, 1,610 were selected in the present study based on the following criteria: (1) They were positive for HBsAg (radioimmunoassay, Dainabot, Tokyo, Japan) and negative for anti-HCV (third-generation enzyme immunoassay, Chiron, CA). (2) They were free of HCC at the initial visit. (3) HBV hepatitis was assessed as less than severe at the initial visit, in order to minimize the potential effects of high inflammatory activity. Severe hepatitis was defined as serum transaminase level of $\geq$300 IU/L, and/or total bilirubin level of $\geq$3.0 mg/dl. (4) They had not received antiviral therapy in the past (e.g., interferon and/or nucleot(s)ide analogs) at the initial visit. (5) They underwent examination of the AFP level (upper limit of normal, 10 μg/L) at the initial visit. Furthermore, the HBsAg level, HBV core-related antigen (HBcrAg) level, and HBV DNA were also assayed using stored frozen sera obtained at the initial visit. (6) They were free of coinfection with human immunodeficiency virus. (7) They were free of other types of chronic liver disease, including hemochromatosis, Wilson disease, primary biliary cirrhosis, alcoholic liver disease, autoimmune liver disease, inherited liver disease including alpha-1 antitrypsin deficiency, and hepatic venous outflow block. (8) They consented to the study.

Table I summarizes the profile and laboratory data at the initial visit of the 1,610 patients included in the present study. They included 1,047 males and 563 females, with a median age of 40 years (range: 18–83 years). The median AFP level was 4 μg/L (range, 1–1,770 μg/L) and the median follow-up time (from the initial visit until the last visit) was 6.0 years (range, 0.0–34.6 years). The study protocol was approved by the Human Ethics Review Committee of Toranomon Hospital.

Laboratory Tests

HBsAg, HBcrAg, and HBV DNA levels were assayed using stored frozen sera obtained at the initial visit. Blood samples were frozen at −80°C within 4 hr of collection and were not thawed until used for testing. Serum HBsAg level was measured using Architect HBsAg QT assay kit (Abbott Laboratories, Tokyo, Japan), which has a lower limit of detection of

TABLE I. Profiles and Laboratory Data at the Initial Visit of 1,610 Patients Infected With HBV

Demographic data	
Number of patients	1,610
Sex (male/female)	1,047/563
Age (years)*	40 (18–83)
Family history of liver disease[a]	836 (51.9%)
Lifetime cumulative alcohol intake (≥500 kg)	112 (7.0%)
Laboratory data*	
Total bilirubin (mg/dl)	0.6 (0.1–2.9)
Aspartate aminotransferase (IU/L)	37 (5–220)
Alanine aminotransferase (IU/L)	48 (5–297)
Albumin (g/dl)	4.2 (1.0–5.6)
Gamma-glutamyl transpeptidase (IU/L)	37 (2–2,370)
Hemoglobin (g/dl)	14.5 (6.9–18.2)
Platelet count (×104/mm^3)	19.1 (2.7–44.7)
Alpha-fetoprotein (μg/L)	4 (1–1,770)
Virological data	
HBeAg (No. of positive)	690 (42.9%)
HBsAg (IU/ml)*	2,845 (0.09 to >125,000)
HBcrAg (log U/ml)*	4.9 (<3.0 to >6.8)
HBV DNA (log copies/ml)*	5.7 (<2.1 to >9.1)
HBV genotype (A/B/C/others/ND)	65/218/1,119/6/202

Data are number and percentages of patients, except those denoted by *, which represent the median (range) values.
[a]Family history of positivity for hepatitis B surface antigen including third-degree relatives.

0.05 IU/ml and upper limit of detection of 250 IU/ml. To expand the upper range from 250 to 125,000 IU/ml, serum samples with the HBsAg levels above the upper range were diluted in a stepwise fashion to 1:20 and 1:500 with Architect diluents using the information supplied by the manufacturer. HBeAg was determined by enzyme-linked immunosorbent assay kit (HBeAg EIA; Institute of Immunology, Tokyo, Japan). Serum HBcrAg level was measured using a Cleia HBcrAg assay kit (Fujirebio, Tokyo, Japan) using a fully automated analyzer system (Lumipulse System; Fujirebio). The cut-off value of HBcrAg was 3.0 log U/ml. HBV DNA was quantified using the Cobas TaqMan HBV v.2.0 (Roche Diagnostics, Tokyo, Japan), which has a dynamic range of 2.1–9.0 log copies/ml.

A commercial kit (HBV Genotype EIA; Institute of Immunology) was used to determine serologically the HBV genotypes using the combination of epitopes expressed on the pre-S2 region product, which is specific to each of the major genotypes.

Follow-Up and Diagnosis of Future Hepatocellular Carcinoma

After the initial visit, patients were followed-up once or three times a month. Imaging studies (ultrasonography, computed tomography, or magnetic resonance imaging) were conducted once or more per year.

Statistical Analysis

Non-parametric tests (Mann–Whitney *U*-test, chi-squared test and Fisher's exact probability test) were used to compare differences between two groups. Correlation analysis was evaluated by the Spearman rank correlation test. The cumulative rate of hepatocarcinogenesis was calculated using the Kaplan–Meier technique; differences between cumulative carcinogenesis curves between groups were tested using the log-rank test. Statistical analyses of the rate of hepatocarcinogenesis according to groups were calculated using the period from the initial visit. Univariate and multivariate logistic regression analyses were used to determine the independent surrogate markers of elevated AFP at the initial visit. The odds ratios (OR) and 95% confidence intervals (95% CI) were also calculated. A two-tailed *P*-value less than 0.05 was considered significant. Variables that achieved statistical significance ($P < 0.05$) on univariate analysis were entered into multiple logistic regression analysis to identify significant independent factors for elevated AFP. Potential surrogate markers of elevated AFP at the initial visit included the following pretreatment variables: age, sex, family history of liver disease, lifetime cumulative alcohol intake, total bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), albumin, gamma-glutamyl transpeptidase (GGT), hemoglobin, platelet count, HBV genotype, HBeAg, HBsAg levels, HBcrAg levels, and HBV DNA levels. Statistical analyses were performed using the Statistical Package for Social Sciences software (SPSS, Inc., Chicago, IL).

RESULTS

Cumulative Rate of Hepatocarcinogenesis According to the AFP Level at the Initial Visit

A total of 1,061 patients naïve to antiviral therapy from the initial visit until the last visit were evaluated for the rate of development of HCC based on the AFP levels at the initial visit. During the follow-up period, HCC was diagnosed in 31 of 905 patients (3.4%) with a low AFP level (≤10 μg/L; below the upper limit of normal) and 37 of 156 patients (23.7%) with a high AFP level (≥11 μg/L) at the initial visit. The cumulative hepatocarcinogenesis rates for patients with low and high AFP levels at the initial visit were 4.7% and 30.2% at the end of 10-year follow-up; 9.1% and 36.5% at the end of 20-year follow-up; and 13.2% and 42.9% at the end of 30-year follow-up, respectively. These results indicate that the rate of hepatocarcinogenesis is significantly higher in patients with HBV infection and high AFP levels than their counterparts with low AFP levels ($P < 0.001$; Log-rank test) (Fig. 1).

HBsAg and AFP Levels at the Initial Visit

Blood samples from all patients were analyzed to determine the relationship between the HBsAg and the AFP levels at the initial visit. The proportions of patients with high AFP levels among those with the HBsAg levels below 500 IU/ml, from 500 to 1,999 IU/ml, from 2,000 to 6,999 IU/ml, from 7,000 to 24,999 IU/ml, and above 25,000 IU/ml were 12.6% (42 of 333 patients), 26.7% (89 of 333), 22.6% (94 of 416), 10.4% (29 of 278), and 6.4% (16 of 250), respectively (Fig. 2A). The relationship between the HBsAg and

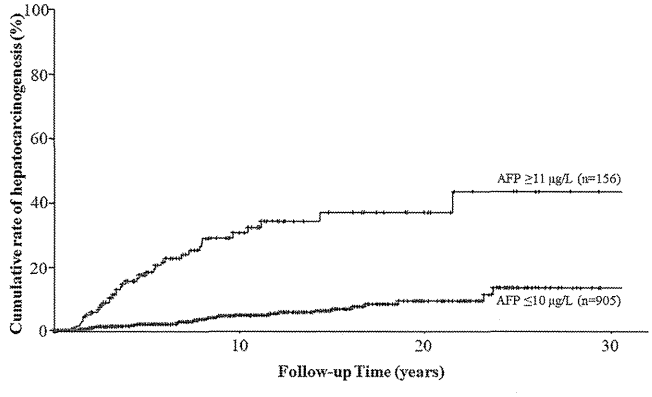

Fig. 1. Cumulative rate of hepatocarcinogenesis according to the AFP level at the initial visit in patients naïve to antiviral therapy from the initial visit until the last visit. The rate of hepatocarcinogenesis was significantly higher in patients with high AFP levels (≥11 μg/L) than in those with low levels (≤10 μg/L) at the initial visit ($P < 0.001$; Log-rank test).

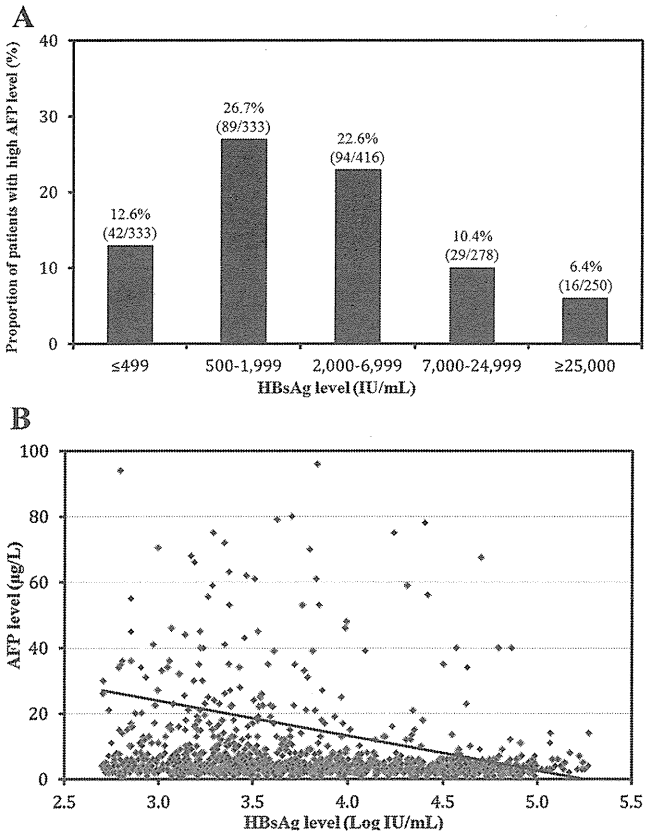

Fig. 2. **A**: Proportions of patients with the high AFP levels (≥11 μg/L) at the initial visit, stratified according to the HBsAg levels. Patients with the HBsAg levels above 500 IU/ml included a significantly lower proportions of patients with the high AFP levels and the HBsAg levels above 7,000 IU/ml (8.5%) than those with the HBsAg levels below 7,000 IU/ml (24.4%) ($P < 0.001$). **B**: Analysis of data of patients with the HBsAg levels above 500 IU/ml at the initial visit, showed a significant negative correlation between logarithmically transformed HBsAg and AFP levels ($r = -0.225$, $P < 0.001$).

the AFP levels at the initial visit suggested the presence of two distinct populations within the study group. Especially, in 1,277 patients with the HBsAg levels above 500 IU/ml, a significantly smaller proportion of patients with high AFP levels were noted among those with HBsAg of more than 7,000 IU/ml (8.5%) than those with the HBsAg levels less than 7,000 IU/ml (24.4%) ($P < 0.001$). Furthermore, the HBsAg levels correlated negatively but significantly with the AFP levels ($r = -0.225$, $P < 0.001$) (Fig. 2B).

The HBsAg Levels and the Platelet Count at the Initial Visit

Blood samples from all patients were analyzed to determine the relationship between the HBsAg levels and the platelet count at the initial visit. The median platelet counts among patients with the HBsAg levels below 500 IU/ml, from 500 to 1,999 IU/ml, from 2,000 to 6,999 IU/ml, from 7,000 to 24,999 IU/ml, and above 25,000 IU/ml were $19.1 \times 10^4/mm^3$, $17.2 \times 10^4/mm^3$, $18.0 \times 10^4/mm^3$, $20.9 \times 10^4/mm^3$, and $21.2 \times 10^4/mm^3$, respectively (Fig. 3A). The relationship between the HBsAg levels and the platelet count at the initial visit also suggested the presence of two distinct populations within the study group. Especially, in 1,277 patients with the HBsAg levels of more than 500 IU/ml, significantly higher platelet counts were noted among those with the HBsAg levels of more than 7,000 IU/ml (the median platelet count; $21.0 \times 10^4/mm^3$) than those with the HBsAg levels less than 7,000 IU/ml (the median platelet count; $17.6 \times 10^4/mm^3$) ($P < 0.001$). Furthermore, the HBsAg

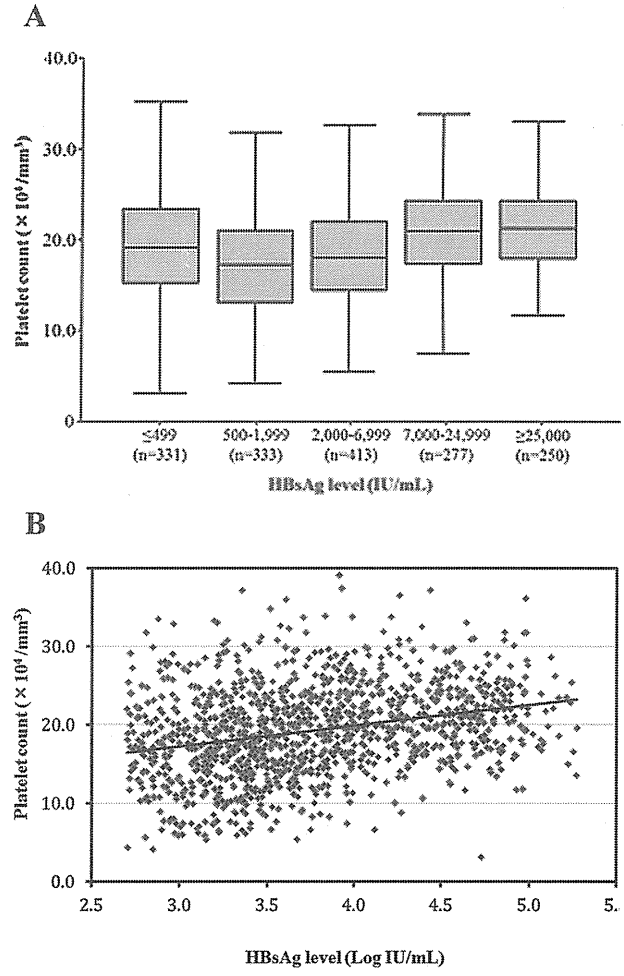

Fig. 3. **A**: The platelet count at the initial visit, stratified according to the HBsAg levels. Bars within the boxes indicate the median platelet count. The boxes denote the 25th to 75th percentiles, the lower and upper bars the 10th and 90th percentiles, respectively. Among patients with the HBsAg levels above 500 IU/ml at the initial visit, those with the HBsAg levels above 7,000 IU/ml had significantly higher platelet count (the median platelet count; $21.0 \times 10^4/mm^3$) compared to those with the HBsAg levels below 7,000 IU/ml (the median platelet count; $17.6 \times 10^4/mm^3$) ($P < 0.001$). **B**: Among patients with the HBsAg levels above 500 IU/ml at the initial visit, logarithmically transformed the HBsAg levels correlated significantly with the platelet count ($r = 0.293$, $P < 0.001$).

levels correlated significantly and positively with the platelet count ($r = 0.293$, $P < 0.001$) (Fig. 3B).

Clinical Profiles and Laboratory Data According to the HBsAg Level at the Initial Visit

Table II summarizes the clinical profiles and laboratory data according to the HBsAg level at the initial visit of 1,610 patients infected with HBV. Patients with the HBsAg levels below 500 IU/ml were significantly older and exhibited lower inflammatory activity (lower levels of AST and ALT), and had lower viral levels (they were HBeAg negative and had lower levels of HBcrAg/HBV DNA), compared to those with the HBsAg levels above 500 IU/ml ($P < 0.001$).

Factors Associated With High AFP Levels at the Initial Visit, Stratified According to the HBsAg Levels

Blood samples from all 1,610 patients were analyzed to determine the factors that affect the AFP level at the initial visit. Among 1,277 patients with the HBsAg levels more than 500 IU/ml at the initial visit, high AFP levels were detected in 228 (17.9%) patients. Univariate analysis identified 12 parameters that correlated significantly with a high AFP level at the initial visit. These included age (≥30 years; $P < 0.001$), AST (≥34 IU/L; $P < 0.001$), ALT (≥43 IU/L; $P < 0.001$), albumin (<3.9 g/dl; $P < 0.001$), GGT (≥50 IU/L; $P < 0.001$), total bilirubin (≥1.0 mg/dl; $P < 0.001$), platelet count ($<20.0 \times 10^4/mm^3$; $P < 0.001$), HBV genotype (C; $P < 0.001$), HBsAg levels (<7,000 IU/ml; $P < 0.001$), HBeAg (positive; $P < 0.001$), HBV DNA (≥5.0 log copies/ml; $P < 0.001$), and HBcrAg (≥3.0 log U/ml; $P < 0.001$). Multivariate analysis that included the above variables identified seven factors that influenced independently the elevated AFP level at the initial visit. These included HBsAg level (<7,000 IU/ml; OR 3.69, $P < 0.001$), albumin (<3.9 g/dl; OR 3.09, $P < 0.001$), platelet count ($<20.0 \times 10^4/mm^3$; OR 2.50, $P = 0.001$), GGT (≥50 IU/L; OR 2.28, $P = 0.001$), AST (≥34 IU/L; OR 2.77, $P = 0.003$), HBeAg (positive; OR 2.07, $P = 0.005$), and HBcrAg (≥3.0 log U/ml; OR 5.10, $P = 0.031$) (Table III).

Among 333 patients with the HBsAg levels less than 500 IU/ml, a high AFP at the initial visit was detected in 42 (12.6%) patients. Univariate analysis identified nine parameters that correlated significantly with a high AFP level at the initial visit. These included AST (≥34 IU/L; $P < 0.001$), ALT (≥43 IU/L; $P = 0.001$), albumin (<3.9 g/dl; $P < 0.001$), GGT (≥50 IU/L; $P < 0.001$), platelet count ($<20.0 \times 10^4/mm^3$; $P = 0.001$), HBV genotype (C; $P < 0.001$), HBeAg (positive; $P < 0.001$), HBV DNA (≥5.0 log copies/ml; $P = 0.001$), and HBcrAg (≥3.0 log U/ml; $P < 0.001$). Multivariate analysis that included the above variables identified three factors that influenced independently the elevated AFP level at the initial visit. These included albumin (<3.9 g/dl; OR 12.8, $P < 0.001$), GGT (≥50 IU/L; OR 6.95, $P = 0.002$), and HBcrAg (≥3.0 log U/ml; OR 5.62, $P = 0.010$) (Table III).

Factors Associated With High AFP Levels at the Initial Visit According to the HBsAg Levels in Patients With Low Transaminase Levels

To minimize the effect of inflammatory activity, we examined the data of 618 (among 1,610 patients) who

TABLE II. Profiles and Laboratory Data of Patients Infected With HBV According to the HBsAg Level at the Initial Visit

	HBsAg <500 IU/L	HBsAg ≥500 IU/L	*P*
Demographic data			
Number of patients	333	1,277	
Sex (male/female)	227/106	820/457	NS
Age (years)*	49 (18–75)	38 (18–83)	<0.001
Family history of liver disease[a]	130 (39.0%)	706 (55.3%)	<0.001
Lifetime cumulative alcohol intake (≥500 kg)	32 (9.6%)	80 (6.3%)	0.037
Laboratory data*			
Total bilirubin (mg/dl)	0.7 (0.2–2.9)	0.6 (0.1–2.9)	0.033
Aspartate aminotransferase (IU/L)	29 (12–175)	40 (5–220)	<0.001
Alanine aminotransferase (IU/L)	32 (7–289)	56 (5–297)	<0.001
Albumin (g/dl)	4.2 (1.1–5.6)	4.2 (1.0–5.5)	NS
Gamma-glutamyl transpeptidase (IU/L)	36 (2–2,370)	38 (4–1,638)	NS
Hemoglobin (g/dl)	14.4 (8.4–17.4)	14.6 (6.9–18.2)	NS
Platelet count ($\times 10^4/mm^3$)	19.1 (2.7–39.6)	19.2 (3.1–44.7)	NS
Alpha-fetoprotein (μg/L)	4 (1–968)	4 (1–1,770)	0.005
Virological data			
HBeAg (No. of positive)	37 (11.1%)	653 (51.1%)	<0.001
HBsAg (IU/ml)*	123 (0.09–498)	4,680 (503 to >125,000)	<0.001
HBcrAg (log U/ml)*	<3.0 (<3.0 to >6.8)	5.9 (<3.0 to >6.8)	<0.001
HBV DNA (log copies/ml)*	3.7 (<2.1 to >9.1)	6.6 (<2.1 to >9.1)	<0.001
HBV genotype (A/B/C/others/ND)	7/104/141/0/81	58/114/978/6/121	<0.001

NS; not significant.
Data are number/percentages of patients, except those denoted by *, which represent the median (range) values.
[a]Family history of positivity for hepatitis B surface antigen including third-degree relatives.

TABLE III. Results of Multivariate Logistic Analysis for Factors Associated With the High AFP Levels at the Initial Visit

Factor	Category	Risk ratio (95%CI)	*P*
Patients with the HBsAg levels above 500 IU/ml (n = 1,277)			
HBsAg (IU/ml)	1: ≥7,000	1	
	2: <7,000	3.69 (2.12–6.41)	<0.001
Albumin (g/dl)	1: ≥3.9	1	
	2: <3.9	3.09 (1.88–5.05)	<0.001
Platelet count ($\times 10^4$/mm^3)	1: ≥20.0	1	
	2: <20.0	2.50 (1.47–4.24)	0.001
Gamma-glutamyl transpeptidase (IU/L)	1: <50	1	
	2: ≥50	2.28 (1.40–3.72)	0.001
Aspartate aminotransferase (IU/L)	1: <34	1	
	2: ≥34	2.77 (1.42–5.39)	0.003
HBeAg	1: Negative	1	
	2: Positive	2.07 (1.24–3.45)	0.005
HBcrAg (log U/ml)	1: <3.0	1	
	2: ≥3.0	5.10 (1.16–22.4)	0.031
Patients with the HBsAg levels below 500 IU/ml (n = 333)			
Albumin (g/dl)	1: ≥3.9	1	
	2: <3.9	12.8 (4.02–41.7)	<0.001
Gamma-glutamyl transpeptidase (IU/L)	1: <50	1	
	2: ≥50	6.95 (2.06–23.5)	0.002
HBcrAg (log U/ml)	1: <3.0	1	
	2: ≥3.0	5.62 (1.51–21.0)	0.010

Low transaminase levels were defined as transaminase levels below the upper limit of normal.

had low transaminase levels (AST ≤33 IU/L and ALT ≤42 IU/L, i.e., below the upper limits of normal) to further determine those factors that determine the high level of AFP at the initial visit. High AFP was detected in 26 (6.1%) patients among 426 with the HBsAg levels above 500 IU/ml and low transaminase levels. Using the data of these patients, univariate analysis identified three parameters that correlated significantly with a high AFP level at the initial visit. These included albumin (<3.9 g/dl; $P = 0.004$), platelet count (<20.0 × 10^4/mm^3; $P = 0.012$), and HBsAg levels (<7,000 IU/ml; $P = 0.004$). Multivariate analysis that included the above variables identified albumin (<3.9 g/dl; OR 3.92, $P = 0.001$) and HBsAg levels (<7,000 IU/ml; OR 4.33, $P = 0.004$) as independent determinants of a high AFP level at the initial visit (Table IV).

Among 192 patients with the HBsAg levels below 500 IU/ml and low transaminase levels, high AFP levels were detected at the initial visit in 12 (6.3%). Univariate analysis identified three parameters that influenced significantly the elevated AFP level at the initial visit. These included albumin (<3.9 g/dl; $P = 0.010$), GGT (≥50 IU/L; $P = 0.011$), and platelet count (<20.0 × 10^4/mm^3; $P = 0.020$). Multivariate analysis that included these variables identified albumin (<3.9 g/dl; OR 7.19, $P = 0.004$) as the only independent determinant of a high AFP level at the initial visit (Table IV).

TABLE IV. Results of Multivariate Analysis for Factors Associated With the High AFP Levels at the Initial Visit

Factor	Category	Risk ratio (95%CI)	*P*
Patients with HBsAg >500 IU/ml and low transaminase levels (n = 426)			
Albumin (g/dl)	1: ≥3.9	1	
	2: <3.9	3.92 (1.71–9.01)	0.001
HBsAg (IU/ml)	1: ≥7,000	1	
	2: <7,000	4.33 (1.58–11.9)	0.004
Patients with HBsAg <500 IU/ml and low transaminase levels (n = 192)			
Albumin (g/dl)	1: ≥3.9	1	
	2: <3.9	7.19 (1.87–27.8)	0.004

Low transaminase levels were defined as transaminase levels below the upper limit of normal.

DISCUSSION

There is little information on the cutoff value of AFP that can be used to predict the future probability of HCC in patients with HBV infection. The present study followed-up patients naïve to antiviral therapy from the initial visit and showed that the rate of hepatocarcinogenesis was significantly higher in those with high AFP levels at the baseline than those with low levels. To our knowledge, the present study is the first to report the hepatocarcinogenesis rate stratified according to the AFP level in patients infected with HBV but free of HCC at the initial visit, based on a large-scale long-term follow-up cohort. The results indicated that patients with high AFP levels at the initial visit are at high risk of HCC, and emphasize the need to determine the factors that could affect the AFP level as surrogate markers of early hepatocarcinogenesis. Previous studies in patients with HCV infection indicated that suppression of the AFP level by treatment with interferon reduced the HCC risk even in those without complete eradication of HCV [Arase et al., 2007; Asahina et al., 2013]. However, there is little

evidence that suppression of the AFP level by antiviral therapy reduces the HCC risk in patients with HBV infection. Further prospective studies are needed to investigate this issue in detail.

In the present study, the relationship between the HBsAg levels and the AFP levels detected at the initial visit suggested the presence of two distinct groups within the study patients. Interestingly, in patients with the HBsAg levels above 500 IU/ml, a significant negative correlation was observed between the HBsAg and the AFP levels, and a significant positive correlation was observed between the HBsAg and the platelet count. Previous studies indicated that high serum AFP levels correlated with liver fibrosis Stage 3 and 4 [Bayati et al., 1998; Chu et al., 2001; Hu et al., 2002, 2004], and that lower thrombocytopenia was closely associated with advanced liver disease [Ikeda et al., 2009; Akuta et al., 2012]. Considered together, these results emphasize the importance of hyper-α-fetoproteinemia and thrombocytopenia in the prediction of severe liver fibrosis, respectively. Based on the present results and the recent reports suggesting the potential correlation between the HBsAg level and the stage of liver fibrosis [Seto et al., 2012; Martinot-Peignoux et al., 2013], it is possible that HBsAg levels could correlate with the stage of fibrosis in patients with the HBsAg levels above 500 IU/ml. Further studies are needed to determine the value of hyper-α-fetoproteinemia in patients with low and high HBsAgemia.

In addition to the HBsAg level, multivariate analysis also identified HBcrAg as another viral factor that influenced independently the AFP level at the baseline. HBcrAg comprises HBcAg, HBeAg and a 22-kDa precore protein coded with the precore/core gene [Kimura et al., 2002, 2005]. Previous studies reported a significant correlation between serum HBcrAg concentrations and intrahepatic levels of covalently closed circular DNA (cccDNA) [Wong et al., 2007; Suzuki et al., 2009]. Other studies indicated that HBcrAg is a useful predictor of HCC during antiviral therapy [Kumada et al., 2013], and post-treatment recurrence of HCC during antiviral therapy [Hosaka et al., 2010]. The present study, based on patients naïve to antiviral therapy showed that high serum HBcrAg concentrations also correlated with high AFP at the initial visit. This is the first report demonstrating the potential usefulness of HBcrAg as a surrogate marker for early hepatocarcinogenesis.

The impact of the HBsAg level on hepatocarcinogenesis is not clear at this stage. In this study, the effect of the HBsAg levels at the initial visit on HCC was assessed in 1,061 consecutive antiviral therapy-naive patients infected with HBV. Analysis of data of 794 patients with the HBsAg levels above 500 IU/ml at the initial visit (after exclusion of patients on antiviral therapy) showed a significantly lower cumulative HCC rate in patients with the HBsAg levels above 7,000 IU/ml than those with levels below 7,000 IU/ml ($P < 0.001$, Log-rank test, Fig. 4). This result suggests that HBsAg levels at the baseline do not only influence AFP, but also play a role in hepatocarcinogenesis. Further studies need to be performed to determine the pathomechanisms of HBsAg in hepatocarcinogenesis.

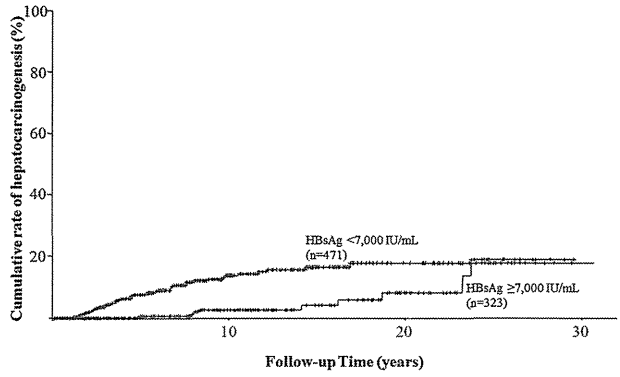

Fig. 4. Cumulative rate of hepatocarcinogenesis stratified according to the HBsAg levels at the initial visit in patients naïve to antiviral therapy from the initial visit until last visit. In a preliminary study based on 794 patients with the HBsAg levels above 500 IU/ml at the initial visit, the cumulative hepatocarcinogenesis rate for patients with the HBsAg levels more than 7,000 IU/ml was significantly lower than for those with levels below 7,000 IU/ml ($P < 0.001$; Log-rank test).

The present study has certain limitations. First, the study did not examine the effects of other genotypes, apart from HBV genotype B or C. Second, the study population was limited to Japanese and did not include other races, and thus generalization of the results to other races cannot be made based on the results. Third, the study did not investigate the effects of antiviral therapy (interferon and/or nucleot(s)ide analogs) on the outcome since such therapy suppressed the AFP levels and thus reduce the risk of HCC in patients with HBV infection.

In conclusion, the present studies demonstrated that the HBsAg level seem to influence the AFP levels and can be used as a surrogate marker for early hepatocarcinogenesis in patients with hepatitis B viral infection.

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Long-Term Entecavir Treatment Reduces Hepatocellular Carcinoma Incidence in Patients With Hepatitis B Virus Infection

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Chronic hepatitis B virus (HBV) infection leads to cirrhosis and hepatocellular carcinoma (HCC). Antiviral agents are thought to reduce HCC development, but agents such as lamivudine (LAM) have a high rate of drug resistance. We compared the incidence of HCC in 472 entecavir (ETV)-treated patients and 1,143 nontreated HBV patients (control group). Propensity score matching eliminated the baseline differences, resulting in a sample size of 316 patients per cohort. The drug mutation resistance was 0.8% (4/472) in the ETV group. The cumulative HCC incidence rates at 5 years were 3.7% and 13.7% for the ETV and control groups, respectively ($P < 0.001$). Cox proportional hazard regression analysis, adjusted for a number of known HCC risk factors, showed that patients in the ETV group were less likely to develop HCC than those in the control group (hazard ratio: 0.37; 95% confidence interval: 0.15-0.91; $P = 0.030$). Both cohorts were applied in three previously reported risk scales and risk scores were generated based on age, gender, cirrhosis status, levels of alanine aminotransferase, hepatitis B e antigen, baseline HBV DNA, albumin, and bilirubin. The greatest HCC risk reduction occurred in high-risk patients who scored higher on respective risk scales. In sub analyses, we compared treatment effect between nucleos(t)ide analogs, which included matched LAM-treated patients without rescue therapy (n = 182). We found HCC suppression effect greater in ETV-treated ($P < 0.001$) than nonrescued LAM-treated ($P = 0.019$) cirrhosis patients when they were compared with the control group. *Conclusion*: Long-term ETV treatment may reduce the incidence of HCC in HBV-infected patients. The treatment effect was greater in patients at higher risk of HCC. (HEPATOLOGY 2013;58:98-107)

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More than 2 billion people worldwide have been exposed to hepatitis B virus (HBV) and about 350 million people are chronically infected, the majority of whom are in Asia (75%). The prevalence of HBV in Japan is 0.8%, which is lower than other Asian countries such as Taiwan (>10%) and China.[1-3] As chronic HBV infection leads to cirrhosis and hepatocellular carcinoma (HCC), published studies have shown that up to 25% of chronically infected patients eventually die of liver cirrhosis or HCC.[4]

A large-scale longitudinal epidemiologic study has shown that a patient's baseline HBV DNA level is an independent predictor for the development of HCC.[5] Studies have begun to show that treatment to decrease HBV DNA reduces the risk of HCC development in HBV patients with cirrhosis or advanced fibrosis or in chronic HBV patients.[6,7]

Within the past 10 years, new antiviral therapies, including nucleos(t)ide analogs (NAs), have been approved and were successful in suppressing circulating serum viral loads. Studies that have examined the relationship between NA therapy and HCC almost exclusively used older drugs such as lamivudine and/or adefovir. Although results of long-term studies showed the importance of antiviral suppression, HCC risk among patients treated by newer NAs remains inconclusive. Entecavir (ETV) is a relatively new antiviral NA that has proved effective in suppressing HBV

Abbreviations: ALT, alanine aminotransferase; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; ETV, entecavir; HBeAg, hepatitis B e antigen; HBV DNA, hepatitis B virus deoxyribonucleic acid; HR, hazard ratio; NA, nucleos(t)ide analogs; PS, propensity score; ROC, receiver operating characteristic curve.

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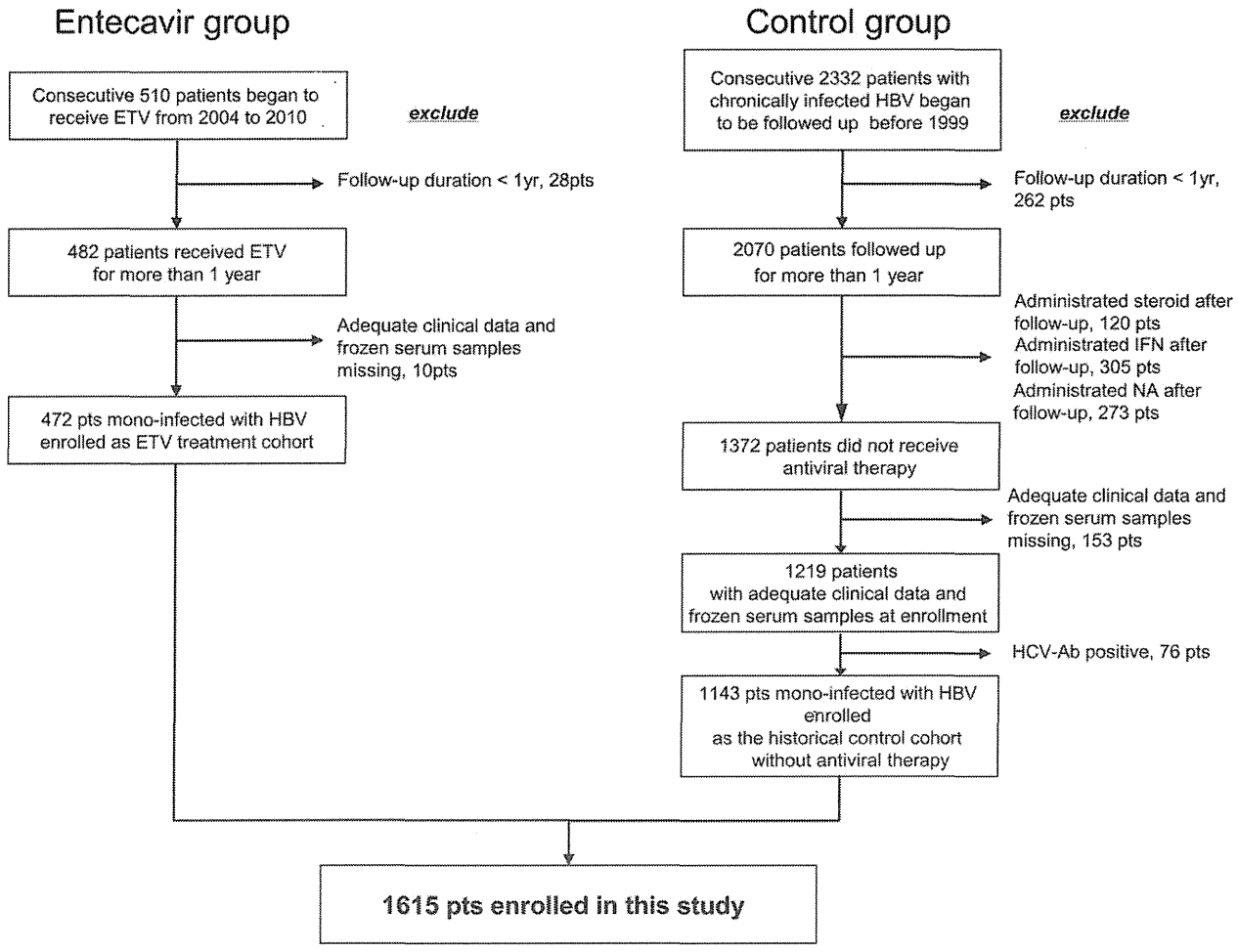

Fig. 1. Entecavir-treated and nontreated cohorts. ETV, entecavir; HBV, hepatitis B virus; IFN, interferon; NA, nucleos(t)ide; HCV-Ab, anti-hepatitis C virus antibody.

DNA replications with minimal drug resistance.[8,9] In this study we examined whether long-term ETV treatment would reduce HCC risk in HBV-infected patients when compared with NA-naïve patients.

Patients and Methods

Patients and Design. From 2004 to 2010, we consecutively recruited 510 patients treated with 0.5 mg ETV (ETV group); the ETV group was compared with a retrospective cohort of 2,332 NA-naïve, HBV-infected patients (control group).

These patients were chronically monoinfected with HBV and were confirmed as hepatitis B s antigen (HBsAg)-positive for at least 6 months. As a general rule, ETV was initiated in a patient who had both abnormal alanine aminotransferase (ALT) levels (defined as ALT $\geq$45) and elevated HBV DNA levels of $\geq$4 log copies/mL. A patient with advanced fibrosis would be treated with ETV if the ALT level was normal; however, a patient without fibrosis or with a normal HBV DNA/ALT level would not be treated with ETV. Among the treated patients, 38 were excluded from the ETV group either because their follow-up period was less than 1 year (n = 28) or because the clinical data or serum samples were incomplete (n = 10). The remaining 472 ETV-treated patients were included in the analysis (Fig. 1). No patient in the ETV group received other NAs before ETV treatment.

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Additional Supporting Information may be found in the online version of this article.

The control group patients were recruited from 1973 to 1999. These patients were NA-naïve at baseline, as no NA therapy had yet been approved. Patients were excluded from the control group if (1) their follow-up duration was less than 1 year (n = 262); (2) corticosteroid withdrawal therapy (n = 120), IFN treatment (n = 305) or NA treatment (n = 273) was initiated during follow-up; (3) clinical data or serum samples were incomplete (n = 153); or (4) patients were found to be positive for anti-hepatitis C virus antibodies (HCV-Ab) (n = 76). The remaining 1,143 patients served as the control population (Fig. 1).

We also made subanalyses to examine the difference of HCC suppression effect between NAs. To make this comparison, we recruited a cohort of 949 consecutive patients from our hospital who were treated with lamivudine (LAM) (September 1995 to September 2007). LAM-treated patients who met the same inclusion criteria as the ETV group, who had no rescue therapy (LAM group, n = 492), were used in the comparison.

We received informed consent from each patient at their entry into the study. Informed consent for the clinical data collection and storage of serum samples were obtained from each patient in the historical control group. The study protocol was in accordance with the ethical guidelines of the Declaration of Helsinki and approved by the Toranomon Hospital Ethics Committee.

Clinical Data Collection and Follow-up. All ETV-treated and untreated patients were followed at 1- to 3-month intervals, during which biochemical and HBV virological markers, blood counts, tumor markers (e.g., alpha-fetoprotein and des-γ-carboxylprothrombin), and cirrhosis and HCC status were monitored. Viral response in the ETV group was defined as a reduction in HBV DNA levels to below 400 copies/mL. Cirrhosis was determined by laparoscopy, liver biopsy, imaging modalities, or portal hypertension. HCC was diagnosed predominantly via imaging, including dynamic computed tomography, magnetic resonance imaging, and/or digital subtraction angiography. When the hepatic nodule did not show typical imaging features, diagnosis was confirmed by fine-needle aspiration biopsy followed by histological examination. Patients were followed until any confirmed HCC diagnosis 1 year after the start of observation (primary outcome) or until the last visit before December 2011. All patients also underwent ultrasonography or helical dynamic computed tomography every 3 to 6 months (cirrhosis patients) or every 6 to 12 months (noncirrhosis patients).

HBV Infection Markers. HBV DNA levels were quantified using the COBAS Amplicor HBV Monitor Test (Roche Diagnostics, Tokyo, Japan), which has a dynamic range of 2.6 to 7.6 log copies/mL, or COBAS TaqMan HBV Test v2.0 (Roche Diagnostics) which has a dynamic range of over 2.1 to 9.0 log copies/mL. HBV DNA of the control group was measured from their stored frozen serum (−80°C) using COBAS TaqMan HBV v.2.0 once at the start of observation. Previous measurements were taken using the old DNA polymerase assay in the control group and thus were not used for comparisons. For the ETV group, drug-resistant mutations were determined from a nested polymerase chain reaction, using a primer specific at the polymerase region in patients who had an HBV DNA relapse of $\geq$1 log copies from nadir. Hepatitis B e antigen; (HBeAg) was determined by enzyme-linked immunosorbent assay with a commercial kit (HBeAg EIA; Institute of Immunology, Tokyo, Japan). A commercial kit (HBV Genotype EIA; Institute of Immunology) was used to serologically determine HBV genotypes using the combination of epitopes expressed on the pre-S2 region product, which is specific for each of the eight major genotypes (A to H).

HCC Incidence by Risk Scores. To examine HCC incidence by risk scores, we applied published HCC risk scales, which are based on the natural course of HCC among HBV-positive patients, to our cohorts. We first searched Medline/PubMed using "hepatitis B," "cancer," and "risk score" as keywords and found four publications in English that used risk-score estimations.[10-13] One article was rejected because we were unable to compute the risk scores with our variables, and therefore we used only the scales indicated by the remaining three publications to generate the risk scores.[13] The risk scales were based on parameters such as age, gender, cirrhosis, levels of ALT, HBeAg, baseline HBV DNA, albumin, and bilirubin. The original risk score formula and the risk score distributions for our two cohorts derived from these formulas are shown in Supporting Table 1. The risk score cutoff points were determined from the following original articles. In Yang et al.'s article,[10] the risk score was derived from 17-point categories. When we applied the scores to our control group, we found that the 12-point scale was at best in detecting a difference in HCC incidence. With that, we examined the HCC suppression treatment effect by dividing the patients into equal halves with 12 points as the cutoff. Yuen et al.[11] divided their cohort in half and found risk scores of 82 as the optimal cutoff point. We also applied the same cutoff point to our cohorts. Wong et al.[12] used their risk scores to categorize their cohort into low-risk, medium-risk, and high-risk groups with respective cutoff points at <4, 4-19, $\geq$20. We also applied the same cutoff points to our cohorts to examine the treatment effect. Cumulative

HCC incidence rates were compared by these risk scores between the ETV and control groups.

Statistical Analysis. Categorical data were compared using chi-square or Fisher's exact tests. Continuous variables with normal distributions were compared using Student's *t* test, and those without normal distributions were compared using the Mann-Whitney *U* test. Cumulative HCC incidence rates were analyzed using the Kaplan-Meier method; patients followed beyond 5 years were censored to better compare the two cohorts because the ETV group had a shorter follow-up period when compared with the historical control group. We compared the cumulative incidence of HCC using the log-rank test, and Cox proportional hazard regression analysis, which was used to assess the variables that were significantly associated with the development of HCC. Deaths before HCC development were censored. Significance was defined as $P < 0.05$ for all two-tailed tests.

We used the propensity score (PS) matching method to reduce significant differences in demographics between the ETV and control groups.[14,15] Using multiple logistic regression analysis, a PS was estimated for all patients treated with ETV.[14] Variables used in the model included age, sex, presence of cirrhosis, HBeAg, HBV DNA< aspartate aminotransferase (AST), ALT, γ-glutamyl transpeptidase; (γ-GTP), bilirubin, albumin, and platelet counts. We performed caliper matching on the PS (nearest available matching). Pairs (ETV and the control group) on the PS logit were matched to within a range of 0.2 standard deviation (SD).[16,17] The PS logit distributions for each cohort showing the overlaps and SD ranges are shown in Supporting Fig. 1. The balance of covariates was measured by their standardized differences. A difference >10% of the absolute value was considered significantly imbalanced.[17] The cohorts were divided into five PS quintiles (Supporting Table 2). We also made subanalyses to examine the difference of HCC suppression effect between NAs by comparing the HCC incidence between propensity score matched ETV- and lamivudine (LAM)-treated patients without a rescue therapy. The LAM-treated patients were derived from consecutive sampling at our institution and were PS matched with ETV group according to the same method described above. Interaction of the subgroups by preexisting cirrhosis or risk scores and ETV treatment were evaluated. $P < 0.10$ was considered statistically significant. Data analysis was performed using IBM SPSS v. 19.0 software (Armonk, NY) and R software v. 2.13 (R Foundation for Statistical Computing, Vienna, Austria; www.r-project.org).

Results

Patient Characteristics. The patient characteristics at the baseline, before PS matching are shown in Table 1. The ETV group was followed for an average of 3.2 years (1,561 person-years), whereas the control group was followed for an average of 9.5 years (12,381 person-years). Before matching, patients in the ETV group and the control group differed significantly in age, gender, genotype, baseline HBV DNA level, and other clinical data. In the ETV group, 421 patients (89%) had HBV DNA (<400 copies/mL) at year 1. Not all patients in the control group were tested for HBV DNA level during follow-up. The drug mutation resistance was 0.8% (4/472). The four patients who had drug mutation did not develop HCC. During follow-up, 12 patients (2.54%) in the ETV group and 144 patients (12.60%) in the control group developed HCC. The incidence rates of HCC for the ETV and the control groups were 76/10,000 patient-years and 116/10,000 patient-years, respectively. During this period, 21 patients in the control group developed liver cirrhosis while no patient developed liver cirrhosis in the ETV group. During the same observation period, there were four deaths in the ETV group and 10 deaths in the control group. We took competing risk into account[18,19] and compared incidence of non-HCC deaths between the cohorts and the results were not different. However, because there were only four patients in the non-HCC deaths in the ETV group (two patients in the PS matched cohort) and 10 patients in the control group (six patients in the PS matched cohort), we considered that it was not meaningful to apply competing risk analysis in our cohorts.

Factors Associated with HCC and Effect of ETV Treatment on HCC Development. To allow a common ground for comparison between the two cohorts, we used PS matching with selected key characteristics and compared the two groups within the same time period of 5 years. The PS matching process resulted in a matched sample size that consisted of 316 patients in each group (Table 1). The PS matching reduced the significant variability of the two cohorts. While five (42%) of the 12 covariates varied by >10% before matching, all covariates differed by <10% of the absolute value after matching (Supporting Fig. 2). In the PS score matched cohort, 10 out of the 231 noncirrhosis patients progressed to liver cirrhosis within the 5 years of observation. The cumulative incidence rates of HCC in the matched ETV groups were 0.7% at year 2, 1.2% at year 3, 2.5% at year 4, and 3.7% at year 5. The cumulative incidence rates of HCC in the