MAJOR ARTICLE

Role of Hepatitis B Virus Precore/Core Promoter Mutations and Serum Viral Load on Noncirrhotic Hepatocellular Carcinoma: A Case-Control Study

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Background. Apart from the presence of liver cirrhosis, hepatitis B virus (HBV) factors have also been shown to play a role in the development of hepatocellular carcinoma (HCC). Studying HBV-related noncirrhotic HCC may help clarify the effect of viral factors.

Methods. In a hospital-based, age- and genotype-matched study, we aimed to determine the role played by basal core promoter (BCP) T1762/A1764 mutation, precore A1896 mutation, and serum viral load in noncirrhotic hepatocarcinogenesis by comparing 44 patients with HBV-related noncirrhotic HCC, 45 patients with chronic hepatitis B, and 42 patients with HBV-related cirrhotic HCC. HBV genotype, precore and BCP mutations, and viral load were determined by molecular assays.

Results. In univariate analysis, statistically significant odds ratios were obtained for male sex ($P = .005$) and BCP T1762/A1764 mutation ($P = .0003$) in patients with noncirrhotic HCC, compared with patients with chronic hepatitis B. By multiple logistic regression analysis, male sex, BCP T1762/A1764 mutation, and viral load $\geqslant 10^5$ copies/mL were independently associated with the risk of noncirrhotic HCC. The virologic characteristics were similar between patients with cirrhotic HCC and those with noncirrhotic HCC.

Conclusions. Our results suggest that BCP T1762/A1764 mutation and higher viral load may be involved in the carcinogenesis of cirrhotic and noncirrhotic HCC.

Hepatitis B virus (HBV) infection is a global health problem, and >350 million people in the world are chronic carriers of the virus [1]. Chronic infection with HBV is a major risk factor for the development of end-stage liver disease and hepatocellular carcinoma (HCC) [2, 3]. The pathogenesis of HCC in HBV infection has been extensively investigated, and multiple factors have been shown to play a role [4]. A major factor is the presence of cirrhosis, a long-term histologic consequence of chronic inflammation and fibrosis, favoring hepatocyte clonal expansion. Also important are viral factors likely involved in the development of HCC [3].

At present, 8 HBV genotypes (A–H) have been identified on the basis of the comparison of complete genomes [5, 6], and most of the genotypes have distinct geographic and ethnic distributions [5–7]. Although the clinical significance of HBV genotypes remains to be firmly settled, it has been shown that patients with HBV genotype C infection are more likely to develop HCC than are those with genotype B infection [8–11]. Because of the spontaneous error rate of viral reverse transcriptase, the HBV genome evolves, undergoing several mutations during the course of persistent infection under the antiviral pressure of the host immunity or specific antiviral therapy [12]. Among HBV mutants, isolates with an A→T transversion at nucleotide 1762 together with a G→A transition at nucleotide 1764 (T1762/A1764) in the basal core promoter (BCP)

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Table 1. Demographic and clinicopathological characteristics of chronic hepatitis B virus (HBV) carriers and patients with noncirrhotic hepatocellular carcinoma (HCC).

Characteristic	Chronic HBV carriers[a] (n = 45)	Patients with noncirrhotic HCC[a] (n = 44)	OR (95% CI)	P
Age, years	44.8 ± 6.1	46.2 ± 9.0	1.02 (0.96–1.08)	.59
Sex				.005
Female	18 (40.0)	6 (13.6)	1.0	
Male	27 (60.0)	38 (86.4)	4.9 (1.6–14.9)	
Family history of HCC	10 (22.2)	11 (25.0)	1.0 (0.9–1.1)	.97
HBV genotype				.32
B	32 (71.1)	31 (70.5)	1.0	
C	13 (28.9)	13 (29.5)	1.7 (0.6–4.6)	
Precore				.07
Wild-type strain	19 (42.2)	11 (25.0)	1.0	
Mutant	26 (57.8)	33 (75.0)	2.3 (0.9–5.9)	
BCP				.0003
Wild-type strain	32 (71.1)	13 (29.5)	1.0	
Mutant	13 (28.9)	31 (70.5)	5.5 (2.2–13.8)	
Viral load, copies/mL				.16
$<10^5$	36 (80.0)	29 (65.9)	1.0	
$\geqslant 10^5$	9 (20.0)	15 (34.1)	2.0 (0.8–5.3)	

NOTE. BCP, basal core promoter; CI, confidence interval; OR, odds ratio.

[a] Data are mean ± SD or no. (%).

(nucleotides 1742–1849) are often present in persons carrying HBV (hereafter, referred to as "carriers") who have chronic hepatitis, fulminant hepatitis, and HCC, and the mutations are present less often in carriers of inactive HBV and in immunosuppressed patients [13–16]. Our previous study clearly demonstrated that HBV carriers with the BCP T1762/A1764 mutant are at increased risk for HCC [17, 18]. Recent studies suggested that, in addition to viral genotype and mutants, high HBV loads are also associated with the progression of chronic liver disease and an increased risk of HCC [19–26].

Because most cases of HCC develop in the presence of liver cirrhosis and because cirrhosis, per se, is an independent risk factor for HCC development, the effect of specific viral factors (e.g., viral mutation or viral load) in hepatocarcinogenesis may be much more evident in noncirrhotic HCC; cirrhotic HCC, however, is rarely encountered clinically. To address this issue, we examined 44 patients with noncirrhotic HCC over a decade and analyzed the contribution of several hepatitis B viral factors to HCC development by comparison with chronic HBV carriers and patients with cirrhotic HCC. Because most Taiwanese HBV carriers acquired HBV infection in their infancy or early childhood, the age of a given patient usually equals the duration of infection. To further exclude the influence of HBV infection duration and to clarify the interplay between HBV genotype and BCP mutation on hepatocarcinogenesis, the patients with noncirrhotic HCC, chronic carriers, and patients with cirrhotic HCC were matched for both age and HBV genotype.

MATERIALS AND METHODS

Patients. From 1990 to 2000, 44 chronic HBV carriers at the gastroenterologic clinics of the National Taiwan University Hospital who had developed noncirrhotic HCC were enrolled. All received surgical resection of HCC without histologic evidence of liver cirrhosis. They included 38 men and 6 women (mean ± SD age, 46.2 ± 9.0 years) and had HBV genotype B infection (n = 31) or genotype C infection (n = 13).

To examine the role of viral factors and to exclude the influence of infection duration on the development of noncirrhotic HCC, during the same period, another 45 age-matched chronic HBV carriers (27 men and 18 women; mean ± SD age, 44.8 ± 6.1 years; genotype B carriage in 32 and genotype C carriage in 13) and 42 patients with HBV-related cirrhotic HCC who also received surgical resection (37 men and 5 women; mean ± SD age, 45.7 ± 6.0 years; genotype B carriage in 26 and genotype C carriage in 16) were selected from our hospital as control subjects. Chronic HBV carriage was defined as positivity for hepatitis B surface antigen for at least 6 months and without ultrasonographic evidence of liver cirrhosis or HCC.

Case patients and control subjects had no serological evidence suggestive of autoimmune chronic liver disease or in-

Table 2. Demographic, clinical, and pathological characteristics of patients with noncirrhotic and cirrhotic hepatocellular carcinoma (HCC).

Value	Patients with noncirrhotic HCC (n = 44)	Patients with cirrhotic HCC (n = 42)	OR (95% CI)	P
Demographic characteristics				.73
Age, years	46.2 ± 9.0	45.7 ± 6.0	…	
Sex			…	.81
Female	6 (13.6)	5 (11.9)	1.0	
Male	38 (86.4)	37 (88.1)	1.2 (0.3–4.2)	
Clinical findings				
Family history of HCC	11 (25.0)	5 (11.9)	0.4 (0.1–1.3)	.13
Child-Pugh class				.17
A	43 (97.7)	36 (90.0)	1.0	
B	1 (2.3)	4 (10.0)	4.8 (0.5–44.7)	
Biochemical values				
Albumin level, mg/dL	4.2 ± 0.4	3.9 ± 0.6	…	.01
Bilirubin level, mg/dL	0.8 ± 0.4	1.1 ± 1.2	…	.24
ALT level, U/L	48.0 ± 43.1	60.4 ± 56.9	…	.26
PT, s	12.8 ± 0.9	13.7 ± 2.0	…	.02
α-Fetoprotein, ng/mL	14,208 ± 28,199	14,336 ± 64,007	…	.99
Pathological findings				
Tumor size, cm	6.6 ± 4.4	4.3 ± 3.4	…	.01
Tumor number			…	.71
Single	33 (75.0)	29 (69.0)	…	
Multiple	11 (25.0)	13 (31.0)	…	
Cell differentiation, Edmonson grade				.75
1	1 (2.3)	1 (2.4)	…	
2	22 (50.0)	26 (61.9)	…	
3	20 (45.4)	13 (30.9)	…	
4	1 (2.3)	2 (4.8)	…	
Fibrosis in nontumor part				
F1	24 (54.5)	…	…	
F2	16 (36.4)	…	…	
F3	4 (9.1)	…	…	
HBV genotype				.40
B	31 (70.5)	26 (61.9)	1.0	
C	13 (29.5)	16 (38.1)	1.5 (0.6–3.6)	
Precore				.71
Wild-type strain	11 (25.0)	12 (28.6)	1.0	
Mutant	33 (75.0)	30 (71.4)	0.8 (0.3–2.2)	
BCP				.16
Wild-type strain	13 (29.5)	7 (16.7)	1.0	
Mutant	31 (70.5)	35 (83.3)	2.1 (0.7–5.9)	
Viral load, copies/mL				.056
$<10^5$	29 (65.9)	19 (45.2)	1.0	
$\geqslant 10^5$	15 (34.1)	23 (54.8)	2.3 (1.0–5.6)	

NOTE. Values expressed as mean ± SD or no. (%). ALT, alanine aminotransferase; BCP, basal core promoter; CI, confidence interval; OR, odds ratio; PT, prothrombin time.

Table 3. Multivariate analysis of possible factors associated with the development of noncirrhotic hepatocellular carcinoma, in comparison with chronic hepatitis B virus carriers.

Factor	OR (95% CI)	*P*
Age, per year	1.0 (0.9–1.1)	.76
Sex, male	4.4 (1.3–15.5)	.02
Precore A1896 mutant	1.9 (0.6–5.5)	.26
Core promoter T1762/A1764 mutant	4.5 (1.7–12.3)	.003
Viral load $\geq 10^5$ copies/mL	2.6 (1.0–11.1)	.049

NOTE. CI, confidence interval; OR, odds ratio.

heritable disorders, such as hemochromatosis or Wilson disease, or history of alcoholism or illicit drug use. None had concomitant hepatitis C virus or hepatitis D virus infection.

Serum samples from each subject were stored at −70°C until use. For patients with histologically verified HCC, serum samples were collected at the time when surgical resection for HCC was performed.

Serological markers. Hepatitis B surface antigen, hepatitis B e antigen, antibodies against hepatitis C virus, and antibodies against hepatitis D virus were tested with commercial kits (Ausria II, IMx HBe 2.0, HCV EIA II, and Anti-Delta, respectively; Abbott Laboratories).

Quantification of HBV DNA. We developed a 1-tube assay for quantification of HBV DNA, as described elsewhere [27]. The HBV DNA concentrations obtained from this quantification assay showed satisfactory consistency with the Amplicor assay (Roche) and the SuperQuant assay (NGI) (Pearson $\rho > 0.99$) in the linear range from 10^2 to 10^{11} copies/mL. The detection limit of HBV DNA is 10^2 copies/mL.

Strict precautions were taken to avoid possible contamination. In addition, reagents were stored in small aliquots. All the pipette tips and Eppendorf tubes were disposable, and the pipettte tips were filtered. Only data that revealed no false-positive results in the negative controls and that were reproducible were used.

Genotyping of HBV. HBV genotype was determined by using the line-probe assay (INNO-LiPA HBV genotyping assay; Innogenetics). In brief, HBV DNA was extracted, and amplification was performed with a polymerase chain reaction assay with the use of primers in the HBV S gene region, as described elsewhere [28]. Samples with detectable HBV DNA after nested polymerase chain reaction were tested by the INNO-LiPA HBV genotyping assay, in accordance with the instructions of the manufacturer.

Determination of precore nucleotide 1896 and BCP dinucleotide 1762/1764. Precore nucleotide 1896 and BCP dinucleotide 1762/1764 were also determined using the line-probe assay (INNO-LiPA HBV PreCore assay; Innogenetics). Except for the primers and reaction strips, the procedure was similar to that for HBV genotyping [14, 29]. The probes were designed to determine the nucleotides at position 1896 (G vs. A) in the precore region and positions 1762 (A vs. T) and 1764 (G vs. A and G vs. T) in the BCP region. Multiple probes were applied on the strip for each motif, taking into account the extensive variability surrounding the specific nucleotide positions assessed by the assay.

Pathological assessment. Grading of the tumor cell differentiation was assessed by the Edmondson classification [30]. The stage of fibrosis was classified, according to the criteria of Desmet et al. [31], from F0 to F4 (F0, no fibrosis; F1, mild fibrosis; F2, moderate fibrosis; F3, severe fibrosis; F4, cirrhosis).

Ethical considerations. The study was performed in accordance with the principles of the 1975 Declaration of Helsinki. The study was approved by the Ethical Committee of the National Taiwan University Hospital, and the serum samples were obtained after receiving informed consent from each patient.

Statistical analysis. Continuous data were expressed as mean ± SD, and the categorical data were expressed as number (percentage). The differences in demographic and clinicopathological features between patients with noncirrhotic HCC and those with cirrhotic HCC or chronic HBV carriers were evaluated by Pearson's χ^2 test, Fisher's exact test, and Student's *t* test where appropriate. Logistic regression analysis was used to assess the influence of each viral factor on the risk of HCC development. A P value of $<.05$ was considered statistically significant.

RESULTS

Demographic and virologic characteristics of subjects. The distribution of age and sex, serum alanine aminotransferase levels, the prevalence of precore A1896 and BCP T1762/A1764 mutants, and the distribution of HBV genotype and serum viral load in patients with noncirrhotic HCC, patients with cirrhotic HCC, and chronic HBV carriers are shown in tables 1 and 2.

Comparison between patients with noncirrhotic HCC and chronic HBV carriers: univariate analysis. When all the demographic and virologic features were compared between chronic HBV carriers and patients with noncirrhotic HCC, we found that statistically significant odds ratios were obtained for male sex ($P = .005$) and BCP T1762/A1764 mutation ($P = .0003$) in patients with noncirrhotic HCC by univariate analysis (table 1).

Comparison between patients with noncirrhotic HCC and chronic HBV carriers: multivariate analysis. To determine the independent contribution of each viral factor to the development of noncirrhotic HCC, multiple logistic regression analysis was performed. In this model, male sex, BCP T1762/A1764 mutation, and viral load $\geq 10^5$ copies/mL were indepen-

dently associated with the development of noncirrhotic HCC (table 3).

Comparison between patients with noncirrhotic HCC and those with cirrhotic HCC. The demographic and virologic characteristics were generally similar between patients with noncirrhotic HCC and those with cirrhotic HCC, except that patients with cirrhotic HCC had significantly lower serum albumin levels (4.2 ± 0.4 mg/dL vs. 3.9 ± 0.6 mg/dL; $P = .01$) and prolonged prothrombin time (12.8 ± 0.9 s vs. 13.7 ± 2.0 s; $P = .02$) and tended to have a higher proportion with serum viral load $\geqslant 10^5$ copies/mL (55% vs. 34%; $P = .056$) (table 2). Histologically, the tumor size was significantly larger in the noncirrhotic HCC group than that in the cirrhotic HCC group (6.6 ± 4.4 cm vs. 4.3 ± 3.4 cm; $P = .01$). The distribution of tumor number and cell differentiation were similar between the 2 groups of patients.

DISCUSSION

The role of host and viral factors in the development of noncirrhotic HCC remains largely unknown. Previous studies have suggested that hepatitis B surface antigen was rarely detected among this group of patients; thus, whether certain hepatitis B viral factors are related to the development of noncirrhotic HCC is ill defined [32–36]. On the other hand, analyzing the viral factors in patients with HCC without evidence of cirrhosis may help dissect the role of HBV itself in hepatocarcinogenesis. In this case-control study, the risk of noncirrhotic HCC development independently increased with male sex, BCP T1762/A1764 mutation, and higher serum viral load. This increased relative risk indicated that viral factors other than the presence of cirrhosis might be involved in hepatocarcinogenesis, such as BCP T1762/A1764 mutation and higher viral load.

Enhancer II and core promoter of the HBV genome control the transcription of the X gene, precore mRNA, and pregenomic RNA [37, 38]. The most well-defined BCP mutant, T1762/A1764, diminishes production of hepatitis B e antigen but increases viral replication [13, 39]. The virulence of the BCP T1762/A1764 mutant remains controversial. In this study, we found that the BCP T1762/A1764 mutation was, indeed, the strongest independent predictor of noncirrhotic HCC development [17]. In addition, the BCP T1762/A1764 mutation affected the risk of developing HCC independent of serum viral load. These findings were consistent with our recent publication [40, 41]. Thus, mechanisms apart from the regulation of HBV replication should be considered in BCP T1762/A1764 mutation–related hepatocarcinogenesis.

In our recent study, the association of HBV genotype C with the development of HCC was found [17]. Furthermore, genotype C tends to have a higher proportion of BCP T1762/A1764 mutation [17]. In addition, the duration of infection may also have an impact on the progression of liver disease and the evolution of viral mutations. To exclude the possible cohort effect and the influence of HBV genotype on BCP mutation, we thus selected age- and genotype-matched controls, and our data suggested that BCP mutation, indeed, independently increased the likelihood of development of noncirrhotic HCC.

The design of our study was retrospective and cross-sectional. Therefore, we cannot exclude the possibility of selection bias and examine other confounding factors. For example, the independent association of male sex with hepatocarcinogenesis in multiple logistic regression analysis could be caused by selection bias. Furthermore, we could not collect serum samples far before the development of HCC; thus, the cause and effect relationship could not be definitely established. Nevertheless, our study was unique in its inclusion of patients with noncirrhotic HCC with detailed clinical, histologic, and virologic data, which allowed us to dissect the role of viral factors in the development of noncirrhotic HCC. In the future, the status of HBV DNA integration in the noncirrhotic HCC and the status of HBx gene expression in the tumor and surrounding nontumor parts should be further examined to support the role of viral factors in hepatocarcinogenesis.

In summary, BCP T1762/A1764 mutation is the strongest viral factor associated with risk of noncirrhotic HCC in HBV carriers. Our findings need to be confirmed in future prospective studies. In addition, further studies regarding HBV-related hepatocarcinogenesis may focus on the structure and function of X protein.

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