MAJOR ARTICLE

Association of Hepatitis B Virus Pre-S Deletions with the Development of Hepatocellular Carcinoma in Chronic Hepatitis B

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Background. We aimed to determine whether hepatitis B virus (HBV) pre-S deletion was an independent factor for the development of hepatocellular carcinoma (HCC).

Methods. Pre-S deletions were determined in HBV isolates from 115 chronic hepatitis B (CHB) patients with HCC. Sixty-nine patients were further matched with 69 CHB patients without HCC for age, sex, hepatitis B e antigen (HBeAg) status, and HBV genotype.

Results. HBV pre-S deletions were clustered mainly in the 3' end of pre-S1 and 5' end of pre-S2 regions. Adjusted for confounding risk factors, patients with HCC had a higher prevalence of HBV with pre-S deletions than did patients without HCC (23 [33.3%] of 69 vs 11 [15.9%] of 69; $P = .018$; odds ratio [OR], 2.64). In particular, only pre-S2 deletions but not pre-S1 deletions were significantly associated with the development of HCC ($P = .020$). A higher prevalence of pre-S deletions was observed in HBV isolates from HCC patients under the age of 50 years than from those older than 50 years (10 [62.5%] of 16 vs 13 [24.5%] of 53; $P = .012$; OR, 5.13). Emergence of de novo pre-S deletions was documented before the development of HCC.

Conclusions. HBV pre-S2 deletions were an independent factor associated with the development of HCC. Its oncogenic role may be more important in young patients with HCC.

Hepatitis B virus (HBV) infection is a global pandemic disease that affects 2 billion people worldwide, around 400 million of whom are chronic HBV carriers. Up to 80% of hepatocellular carcinoma (HCC) is caused by HBV infection. However, the oncogenic mechanisms of HBV remain elusive. The relationship between viral genome mutations and the development of HCC has not been thoroughly investigated.

The HBV pre-S region is located at 5' of open reading frame of the surface gene and is further divided into pre-S1 and pre-S2. Naturally occurring pre-S mutations have been identified frequently during the course of chronic infection, especially at a later stage of the disease [1]. Commonly found pre-S mutations include deletions at the 3' terminus of pre-S1 region, the 5' terminus of pre-S2 region, and the pre-S2 start codon, and point mutations at the pre-S2 start codon. There are several possible hepatocarcinogenic effects of pre-S deletions. Both pre-S1 and pre-S2 mutations have been reported to cause overproduction and accumulation of large hepatitis B surface (LHBs) protein in the endoplasmic reticulum (ER), resulting in significant ER stress that may induce DNA damage and genomic instability and hence a possible role in hepatocarcinogenesis [2]. The transcriptional transactivating capacity of the truncated pre-S2 sequence when integrated into a host genome may be another oncogenic mechanism [3, 4]. Pre-S2 mutations also induce degradation of cyclin-dependent kinase inhibitor [5], leading to uncontrolled cell cycle progression. A high correlation between the expression of pre-S2 mutations and mutation of the tumor suppressor gene p53 has also been observed [2]. Pre-S2 mutations lead to the formation of type II ground glass hepatocytes (GGHs), which correlates with more severe liver diseases, such as cirrhosis and HCC [6].

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Recent studies suggest that HBV pre-S mutations, in particular the pre-S deletions, are associated with the development of HCC [7–10]. Nevertheless, the observed association is essentially based on cross-sectional studies without consideration of other possible confounding factors. Moreover, longitudinal studies are required to determine the association between the development of these mutations and of HCC. Such longitudinal studies may also reveal any changes in the size and location of pre-S deletions during the course of disease progression. We aimed to determine whether HBV pre-S deletions were independently associated with the development of HCC by a case-control study. In addition, we examined the emergence of pre-S deletions prior to the development of HCC by a longitudinal approach.

PATIENTS AND METHODS

Patients and Serum Samples

A total of 115 chronic hepatitis B (CHB) patients with HCC were recruited from the Hepatitis Clinic and Liver Clinic, Queen Mary Hospital, The University of Hong Kong, Hong Kong. These patients had the diagnosis of HCC between the years 2000 and 2008. Ninety-six of them were successfully matched with 96 CHB patients without HCC for age, sex, and hepatitis B e antigen (HBeAg) status. This constituted a population of 192 patients (96 HCC and 96 matched non-HCC patients) for a matched case-control study. Among the 115 HCC patients, 15 of those who carried HBV with pre-S deletions were further studied by examining the pre-S deletions longitudinally prior to the diagnosis of HCC. All patients were positive for hepatitis B surface antigen (HBsAg) for $\geqslant$6 months. The diagnosis of HCC was based on one of the following criteria: (1) positive histology or (2) elevated alpha-fetoprotein levels together with imaging features compatible with HCC by computed tomography, magnetic resonance imaging, or hepatic angiogram. The study was approved by the Institutional Review Board of The University of Hong Kong/Hospital Authority Hong Kong West Cluster, Hong Kong Special Administrative Region.

HBV DNA Extraction

Serum samples of 200 μL were used to extract HBV DNA using the QIAamp DNA Blood Mini Kit (QIAGEN) in accordance with manufacturer's instructions, with a resultant elution volume of 50 μL.

HBV Genotyping

Serologic typing was performed by HBV Genotype enzyme immunoassay (EIA) (Institutes of Immunology) and subsequently validated by phylogenetic analysis.

PCR Amplification of HBV Pre-S Region

Semi-nested polymerase chain reaction (PCR) was used to amplify the entire *pre-S1/S2* gene, as described by Sugauchi et al [11]. All PCR reactions were performed with AmpliTaq Gold DNA Polymerase in the GeneAmp PCR System 9700 (Applied Biosystems). First round PCR was performed in a total volume of 50 μL containing 5 μL extracted HBV DNA as template, 1 X Taq buffer, 1 unit AmpliTaq Gold DNA Polymerase, 1.5 μM $MgCl_2$, 200 μM of each dNTP and 0.2 μM of outer sense primer HBPS1 (5′ CGCAGAAGATCTCAATCTCGG 3′ [nt 2417–2437]) and antisense primer HBPS2 (5′ CGAGTCTA-GACTCTGTGGTA 3′ [nt 256–237]). The conditions for first round PCR were as follows: an initial denaturation step at 95°C for 10 min; 35 cycles of amplification at 94°C, 60°C, and 72°C, each for 1 min; and a final extension step at 72°C for 7 min. Inner primers for the second round PCR were HBPS3 (sense, 5′ GGGTCACCATATTCTTGGGAA 3′ [nt 2814–2834]) and HBPS2, with 2 μL of the first round PCR product as a template. Thirty amplification cycles with the same conditions as the first round were performed, and the products with expected size of 628 bp were detected by electrophoresis in 2% agarose gel stained with ethidium bromide.

Sequence Analysis and Detection of Pre-S Deletions

The PCR products were purified using PCRquick-spin PCR Product Purification Kit (iNtRON Biotechnology) and sequenced bi-directionally with the second round primers using the ABI PRISM BigDye Terminators reaction kit and an ABI PRISM 3730*xl* DNA Analyzer (Applied Biosystems). The MEGA 3.1 program [12] was used to assemble the sense and antisense sequences of each sample into a contig sequence and to align the validated sequences with respect to the wild-type HBV DNA reference sequence (GenBank accession number: AF286594). Pre-S deletions were denoted by the absence of 1 or more nucleotides in the aligned sequences.

Statistical Analysis

Data were analyzed using the Statistical Program for Social Science, version 16.0 for Windows (SPSS). The χ^2 test and the Fisher exact test were used for categorical data. The Student *t* test and the Mann-Whitney *U* test were used for continuous data with normal and skewed distribution, respectively. All tests were 2-tailed, and a *P* value of $<$.05 was considered to be statistically significant.

RESULTS

Demographic Characteristics and Prevalence of HBV Pre-S Deletion Mutations in the Matched Cohort

Table 1 shows the demographic characteristics and the prevalence of HBV pre-S deletion mutations of the matched cohorts. The study population was mostly male (85.4%) and HBeAg-negative (75%). The nucleotide sequence data reported in this article have been submitted to the DDBJ/EMBL/GenBank databases under the accession numbers HQ379433-HQ379624. All HBV genotypes in the patients were either genotype B or C.

Table 1. Demographic Characteristics of Hepatitis B Virus (HBV)-Infected Individuals in 2 Clinical Groups Matched by Age, Sex, and Hepatitis B e Antigen (HBeAg) Status

Variable	Non-HCC (n = 96)	HCC (n = 96)	P
Age, mean ± SD, years	56.8 ± 11.5	56.9 ± 11.5	NS
Age group			
<50 years	21 (21.9)	20 (20.8)	
⩾50 years	75 (78.1)	76 (79.2)	
Sex			NS
Male	82 (85.4)	82 (85.4)	
Female	14 (14.6)	14 (14.6)	
HBeAg status			NS
Positive	24 (25.0)	24 (25.0)	
Negative	72 (75.0)	72 (75.0)	
HBV genotype			.011
B	45 (46.9)	28 (29.2)	
C	51 (53.1)	68 (70.8)	
Pre-S deletion mutant	14 (14.6)	28 (29.2)	.015

NOTE. Data are no. (%) of patients unless otherwise specified. HCC, hepatocellular carcinoma; NS, not significant.

HBV genotype C was more prevalent in the HCC group than in the non-HCC group (68 [70.8%] of 96 vs 51 [53.1%] of 96; P = .011; odds ratio [OR], 2.14; 95% confidence interval [CI], 1.18–3.89).

A total of 42 patients (28 HCC and 14 non-HCC patients) harbored HBV isolates with pre-S deletions. A significantly higher prevalence of HBV pre-S deletions was detected in HCC patients than in non-HCC patients (28 [29.2%] of 96 vs 14 [14.6%] of 96; P = .015; OR, 2.41; 95% CI, 1.12–3.56). A summary of the pre-S deletion mutations and alignment of their amino acid sequences is presented in Table 2 and Figure 1, respectively. Overall, HBV pre-S deletions were clustered mainly in the 3' end of pre-S1 region and in the 5' end of pre-S2 region. Among the 42 HBV isolates with pre-S deletions identified in both clinical groups, 41 (97.6%) were found from male patients, 39 (92.9%) belonged to HBV genotype C, and 32 (76.2%) had the deletions in the pre-S2 region. All pre-S deletions were in-frame with the size of deletion ranging from 3 to 189 bp. Loss of pre-S1 start codon and pre-S2 start codon was identified in 7 (16.7%) and 8 (19.0%) mutations, respectively. A high proportion of the pre-S deletion mutations had deletions in the human serum albumin receptor (25 [60.0%] of 42) and the S promoter (16 [38.1%] of 42), but fewer in the hepatocyte binding site (2 [4.8%] of 42) and CCAAT box (7 [16.7%] of 42).

HBV Genotypes

In our study the initial matching of HCC and non-HCC patients did not include HBV genotype as one of the matching criteria. Previous reports [8, 9] indicate that HBV genotype C but not genotype B may be associated with the development of pre-S deletion mutations. Because the present study found that HBV genotype C was more common than genotype B in HCC patients (P = .011), HBV genotype could be a potential confounding factor for the effect of pre-S deletions on the development of HCC. Applying HBV genotype as an additional matching criterion to the previously matched 96 HCC/non-HCC pairs, there were 69 further matched pairs.

The demographic characteristics of these 69 further matched pairs were the same as the previously matched 96 pairs, in terms of age (mean age, 56 years; 76.8% ⩾ 50 years), sex (87% male), and HBeAg status (negative in 72.5%)(Table 3). Sixty-seven percent of the patients had HBV genotype C. The prevalence of pre-S deletion mutations in the HCC group was significantly higher than in the non-HCC group (23 [33.3%] of 69 vs 11 [15.9%] of 69; P = .018; OR, 2.64; 95% CI, 1.17–5.96).

Deletion Types in the Pre-S Region

To further analyze the HBV pre-S deletions, the mutations were grouped into 3 categories: mutations with pre-S1 deletions only (pre-S1 only), mutations with pre-S2 deletions only (pre-S2 only), and mutations harboring both pre-S1 and pre-S2 deletions (pre-S1+2). All deletion types were found in higher frequencies in the HCC group, compared with the non-HCC group (pre-S1 only, 8.7% vs 5.8%; pre-S2 only, 18.8% vs 5.8%; pre-S1 + 2, 5.8% vs 4.4%). Among the 3 deletion types, mutations with pre-S2 deletions only were found to be statistically associated with the development of HCC (P = .020).

Correlation between Pre-S Deletions and the Matched Parameters

Figure 2 shows the prevalence of pre-S deletion mutations in the 2 clinical groups stratified by HBV genotypes, sex, age, and HBeAg status. Pre-S deletions were more prevalent in patients infected with HBV genotype C than genotype B, regardless of the presence of HCC. The highest prevalence was found in HCC patients with genotype C infection (21 [45.7%] of 46), followed by non-HCC patients with genotype C infection (11 [23.9%] of 46) and HCC patients with genotype B infection (2 [8.7%] of 23). No pre-S deletions were identified in non-HCC patients with genotype B infection. HBV mutations harboring only pre-S2 deletions were found mainly in HCC patients regardless of HBV genotype B/C.

When patients were stratified by sex, HBV pre-S deletions were more commonly isolated in male than female patients, regardless of the presence of HCC (Figure 2). The prevalence of HBV pre-S deletions in male HCC patients (22 [36.7%] of 60) was higher than that in male non-HCC patients (11 [18.3%] of 60). In contrast, a lower prevalence of the HBV pre-S deletions was found in female HCC patients (1 [11.1%] of 9) and none in female non-HCC patients. In male HCC patients with HBV isolates with pre-S deletions, pre-S2 deletions were the predominant type of mutations. The only HBV deletion isolated

Table 2. Pre-S Deletions in 14 Non-Hepatocellular Carcinoma (HCC) and 28 HCC Patients with Chronic Hepatitis B Virus (HBV) Infection

									Deletion in		
Isolate	Liver status	Age/sex	HBeAg	HBV genotype	Deletion type	Deletion size (bp)	Region (nt)	Deletion In-frame	Hepatocytebinding site	S promoter	CCAAT	Human serum albumin receptor
51948	Non-HCC	32M	+	C	Pre-S1	39	3046–3084	Yes	−	+	−	−
100856	Non-HCC	60M	−	C	Pre-S2	54	3217–3270	Yes	−	−	−	+
101118	Non-HCC	58M	+	C	Pre-S2	63	3208–3270	Yes	−	−	−	+
101370	Non-HCC	71M	−	C	Pre-S2 [b]	3	3205–3207	Yes	−	−	−	−
101719	Non-HCC	59M	−	C	Pre-S2	36	3238–3273	Yes	−	−	−	+
102609	Non-HCC	40M	−	C	PreS1[a]	15	2848–2862	Yes	−	−	−	−
106037	Non-HCC	46M	−	C	Pre-S2	12/21	3220–3231/ 3250–3270	Yes/Yes	−	−	−	+
106270	Non-HCC	66M	+	C	Pre-S1	111	3043–3153	Yes	−	+	+	−
106967	Non-HCC	52M	+	C	Pre-S1	111	3070–3180	Yes	−	+	+	−
107504	Non-HCC	58M	+	C	Pre-S1+2 [b]	147	3127–3273	Yes	−	+	+	+
120621	Non-HCC	49M	+	C	Pre-S2	24	3247–3270	Yes	−	−	−	+
121511	Non-HCC	71M	−	C	Pre-S1+2	171/42	2944–3114/ 3214–3255	Yes/Yes	+	+	−	+
123925	Non-HCC	53M	−	C	Pre-S1+2	3/21	3115–3117/ 3250–3270	Yes/Yes	−	+	−	+
132774	Non-HCC	53M	−	C	Pre-S2	54	3217–3270	Yes	−	−	−	+
36122	HCC	68M	+	C	Pre-S1+2	45/57	3034–3078/ 3217–3273	Yes/Yes	−	+	−	+
46906	HCC	76M	−	C	Pre-S2	30	3241–3270	Yes	−	−	−	+
50141	HCC	48F	−	C	Pre-S1+2 [b]	60	3148–3207	Yes	−	+	−	−
60214	HCC	46M	+	C	Pre-S2	51	3220–3270	Yes	−	−	−	+
75976	HCC	57M	−	C	Pre-S1 [a]	183	3016–3198	Yes	−	+	+	−
HCC100	HCC	35M	−	C	Pre-S2	12	3256–3267	Yes	−	−	−	+
HCC140	HCC	19M	+	C	Pre-S1 [a]	18	2848–2865	Yes	−	−	−	−
HCC160	HCC	51M	−	B	Pre-S2 [b]	3	3205–3207	Yes	−	−	−	−
HCC176	HCC	58M	+	B	Pre-S1+2 [a,b]	21/144	2848–2868/ 3130–3273	Yes/Yes	−	+	+	+
HCC245	HCC	44M	+	C	Pre-S1	111	3070–3180	Yes	−	+	+	−
HCC252	HCC	54M	−	C	Pre-S2	51	3217–3267	Yes	−	−	−	+
HCC277	HCC	50M	−	C	Pre-S2	3	3268–3270	Yes	−	−	−	+
HCC285	HCC	56M	+	C	Pre-S2	51	3217–3267	Yes	−	−	−	+
HCC31	HCC	71M	−	C	Pre-S2	54	3217–3270	Yes	−	−	−	+
HCC438	HCC	65M	+	C	Pre-S1	3	3115–3117	Yes	−	+	−	−
HCC478	HCC	57M	+	C	Pre-S1+2 [a,b]	18/111	2848–2865/ 3109–3219	Yes/Yes	−	+	+	−
HCC48	HCC	66M	−	C	Pre-S2	36	3220–3255	Yes	−	−	−	+
HCC69	HCC	49M	−	B	Pre-S2 [b]	3	3205–3207	Yes	−	−	−	−
NHCC1	HCC	33M	+	C	Pre-S2	24	3247–3270	Yes	−	−	−	+
NHCC11	HCC	59M	−	C	Pre-S2	51	3220–3270	Yes	−	−	−	+
NHCC13	HCC	61M	+	C	Pre-S1 [a]	21	2848–2868	Yes	−	−	−	−
NHCC25	HCC	54M	+	C	Pre-S1+2	33/21	3046–3078/ 3217–3237	Yes/Yes	−	+	−	−
R1227	HCC	60M	−	C	Pre-S2	51	3220–3270	Yes	−	−	−	+
R2290	HCC	31M	−	C	Pre-S1	189	2941–3129	Yes	+	+	−	−
R406	HCC	37M	−	C	Pre-S1+2 [a,b]	18/102	2848–2865/ 3151–3252	Yes/Yes	−	+	−	−
TT185	HCC	40M	−	C	Pre-S2	24	3247–3270	Yes	−	−	−	+
TT262	HCC	78M	−	C	Pre-S2	30	3238–3267	Yes	−	−	−	+
TT431	HCC	61M	−	C	Pre-S2	48	3223–3270	Yes	−	−	−	+

NOTE. HBeAg, hepatitis B e antigen.

[a] Loss of preS1 start codon;

[b] Loss of preS2 start codon.

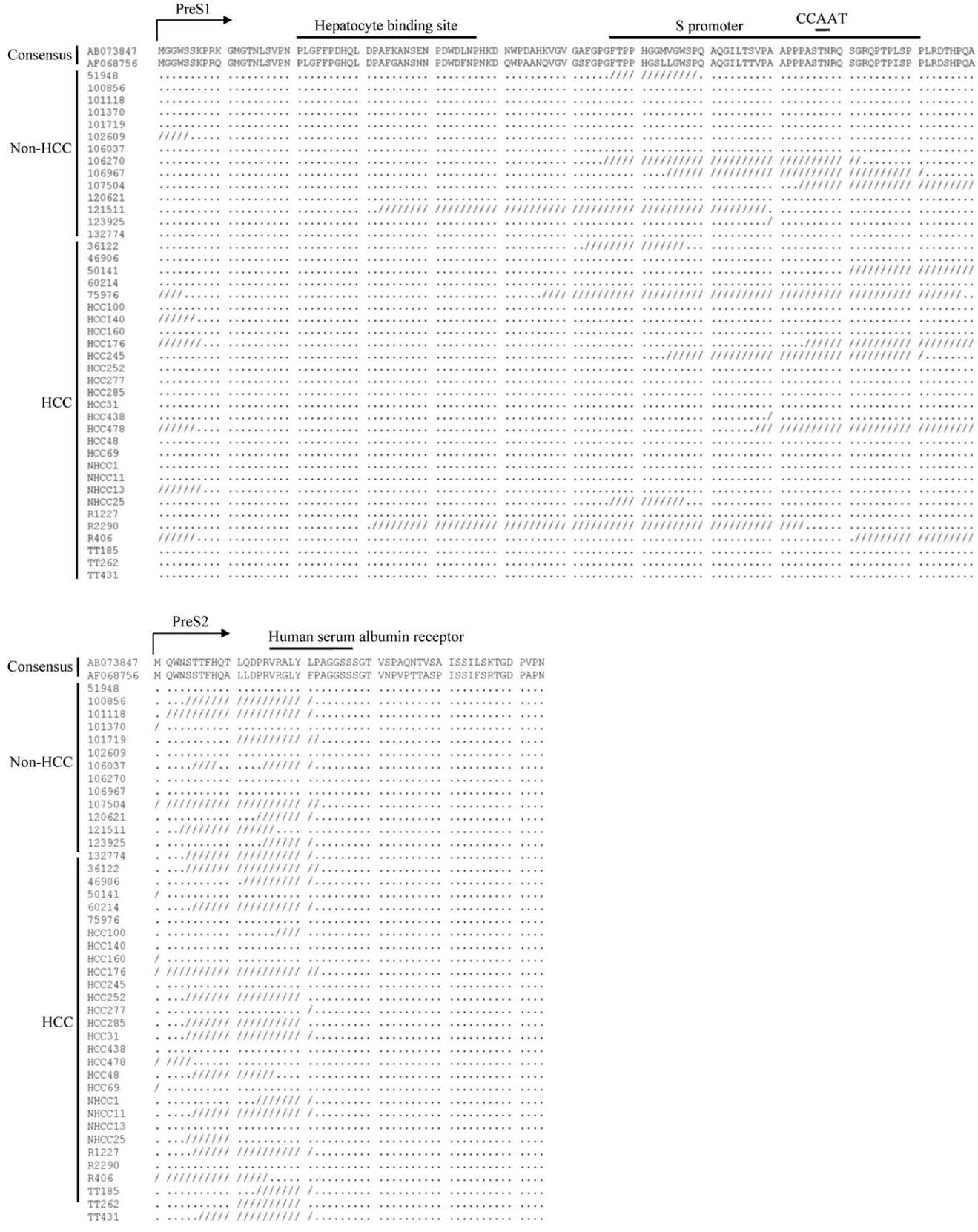


Figure 1. Alignment of amino acid sequences with pre-S deletion from 14 non-HCC and 28 HCC patients. The wild type consensus sequences are represented by AB073847 (genotype B) and AF068756 (genotype C). Dots represent amino acids that may or may not be identical to the consensus sequences. Slashes represent deletion of amino acids. The names of the isolates correspond to those in Table 2. The start sites of preS1 (aa 1) and preS2 (aa 120), hepatocyte binding site (aa 21 — 47), S promoter (nt 3045 — 3180), CCAAT box (nt 3137 — 3141), and human serum albumin receptor (aa 17 — 28) are marked correspondingly at the top of the alignment. aa, amino acid; HCC, hepatocellular carcinoma; nt, nucleotide.

Table 3. Demographic Characteristics of Hepatitis B Virus (HBV)-Infected Individuals in 2 Clinical Groups Matched by Age, Sex, Hepatitis B e Antigen (HBeAg) Status, and HBV Genotype

Variable	Non-HCC (n = 69)	HCC (n = 69)	P
Age, mean ± SD, years	56.0 ± 11.7	56.1 ± 11.7	NS
Age group			
<50 years	16 (23.2)	16 (23.2)	
⩾50 years	53 (76.8)	53 (76.8)	
Sex			NS
Male	60 (87.0)	60 (87.0)	
Female	9 (13.0)	9 (13.0)	
HBeAg status			NS
Positive	19 (27.5)	19 (27.5)	
Negative	50 (72.5)	50 (72.5)	
HBV genotype			NS
B	23 (33.3)	23 (33.3)	
C	46 (66.7)	46 (66.7)	
Pre-S deletion mutant	11 (15.9)	23 (33.3)	.018

NOTE. Data are no. (%) of patients unless otherwise specified. HCC, hepatocellular carcinoma; NS, not significant.

from a female HCC patient was a deletion spanning both pre-S1 and pre-S2 regions.

Figure 2 also shows the prevalence of pre-S deletions stratified by age groups. A considerably higher prevalence of HBV pre-S deletions was found in HCC patients aged <50 years (10 [62.5%] of 16), whereas the prevalence in other categories was lower (range, 15.1%–24.5%).

Within the HCC group, HBV isolates from HBeAg-positive patients had a higher prevalence of pre-S deletions than did those from HBeAg-negative patients (9 [47.4%] of 19 vs 14 [28.0%] of 50), as shown in Figure 2d. A higher prevalence of pre-S deletions in HBV isolates was also observed in HBeAg-positive patients, compared with the prevalence of pre-S deletions in HBV isolates in HBeAg-negative patients, in the non-HCC group (5 [26.3%] of 19 vs 6 [12.0%] of 50). HBV pre-S deletions harboring only pre-S1 deletions were more commonly found in HBeAg-positive patients in both the non-HCC group and HCC group, whereas those harboring only pre-S2 deletions were more commonly found in HBeAg-negative patients in both the non-HCC group and HCC group.

Longitudinal Cohort

Of the 29 HCC patients found to have HBV isolates with pre-S deletions in the matched case-control study, 15 had their serum samples available for testing at an interval of 1–7 years prior to the diagnosis of HCC (6 patients had 2 samples, and 9 patients had 1 sample). Of these 15 HCC patients, 4 (26.7%) harbored HBV with only pre-S1 deletions, 7 (46.7%) harbored HBV with only pre-S2 deletions, and 4 (26.7%) harbored HBV with both pre-S1 and pre-S2 deletions. Four (26.7%) and 3 (20.0%) patients had HBV isolates with pre-S deletions with loss of the start codon in the pre-S1 and pre-S2 region, respectively. All deletions were in-frame, and the deletion size ranged from 3 to

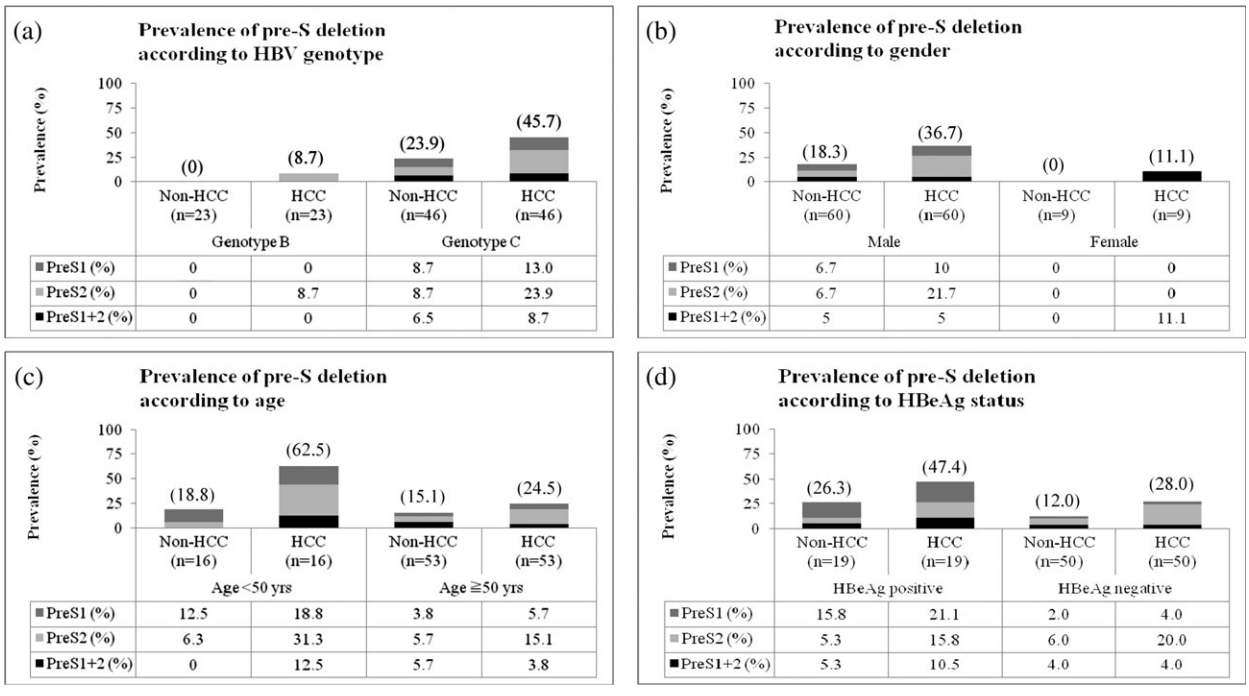


Figure 2. Prevalence of different types of pre-S deletions in 2 clinical groups according to (a) hepatitis B virus (HBV) genotype, (b) gender, (c) age group, and (d) hepatitis B e antigen (HBeAg) status. The number in parentheses on each column represents the overall prevalence of pre-S deletion in that category.

183 bp. Deletions were frequently found in the S promoter region (7 [46.7%] of 15) and the human serum albumin receptor region (8 [53.3%] of 15) but were less commonly found in the hepatocyte binding site (1 [6.7%] of 15) and the CCAAT box (4 [26.7%] of 15).

The status of HBV pre-S deletions is depicted in Figure 3. Five patients (patients 1–5) (33.3%) had no HBV pre-S deletions isolated 1–4.5 years before the diagnosis of HCC, whereas 10 patients (patients 6–15) (66.7%) had HBV pre-S deletions isolated at least 1–6.5 years before the diagnosis of HCC.

The size and location of pre-S deletions remained the same during the course of HCC development in all patients except with regard to the isolate from patient 6, where the pre-S1 deletion was absent in the isolate 3.5 years before the development of HCC but was detected from the later serum samples.

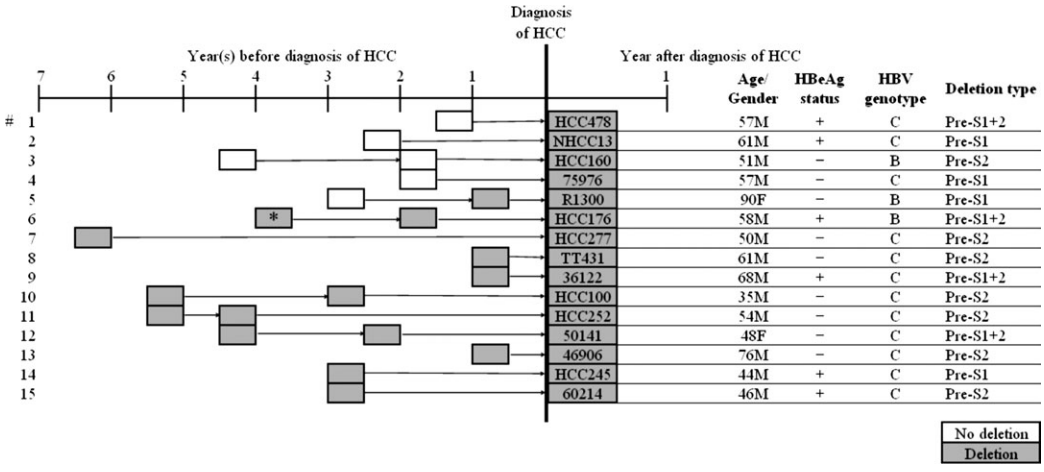
DISCUSSION

In the present matched case-control study, the prevalence of HBV pre-S deletions in HCC and non-HCC patients was 29.2% and 14.6%, respectively, with an overall prevalence of 21.9% (Table 1). Our findings were in accordance with those reported in other Asian countries [11, 13].

Although there is evidence for the association of pre-S deletions with the development of HCC, previous reports were essentially based on cross-sectional studies without taking various possible confounding factors into considerations. Older age, male sex and infection with HBV genotype C (compared with genotype B) are risk factors for HCC [14, 15]. Older age and HBV genotype C are associated with the presence of pre-S deletions [7, 8, 10, 11]. The relationship between HBeAg status and pre-S deletions remains unclear. The potential confounding effect of these factors should be considered in studying the relationship between pre-S deletions and HCC.

In the present study, HCC patients and non-HCC CHB patients were matched for age, sex, HBeAg status, and HBV genotype. Without the potential effects of these factors, the prevalence of pre-S deletions in HCC patients was demonstrated to be significantly higher than that in non-HCC patients ($P = .018$), confirming the association of pre-S deletions with the development of HCC (Table 3). In addition, there was a remarkably higher prevalence of pre-S deletions in HCC patients aged < 50 years, compared with older HCC patients (62.5% vs 24.5%) (Figure 2). Pre-S deletions may play an even more important role in hepatocarcinogenesis in younger patients with HCC.

In the present study, pre-S2 deletions were detected in a higher frequency than pre-S1 deletions in HBV isolates among HCC patients, which is consistent with the findings from other studies [7, 16]. This suggests the higher oncogenic potential of pre-S2 deletions, compared with that of pre-S1 deletions. However, a higher frequency of HBV isolates with pre-S1 deletions than pre-S2 deletions from HCC patients has been reported in 2 Korean studies [13, 17]. The discrepancy may be related to the different proportions of HBeAg-positive and HBeAg-negative patients. Studies have shown that HBV pre-S2 deletions were more frequently found in HBeAg-negative patients than HBeAg-positive patients irrespective of the presence or absence of HCC [11, 13]. It has been strongly suggested that pre-S2 deletions respond more actively to immunologic pressure because of the fact that pre-S2 region contains numerous highly immunogenic T- and B-cell epitopes [18]. Another possible explanation for the difference in the prevalence of pre-S1 and pre-S2 deletions in HCC patients between the Korean study and our study is that almost all Korean CHB patients were infected by HBV genotype



#		Age/ Gender	HBeAg status	HBV genotype	Deletion type
1	HCC478	57M	+	C	Pre-S1+2
2	NHCC13	61M	+	C	Pre-S1
3	HCC160	51M	−	B	Pre-S2
4	75976	57M	−	C	Pre-S1
5	R1300	90F	−	B	Pre-S1
6	HCC176	58M	+	B	Pre-S1+2
7	HCC277	50M	−	C	Pre-S2
8	TT431	61M	−	C	Pre-S2
9	36122	68M	+	C	Pre-S1+2
10	HCC100	35M	−	C	Pre-S2
11	HCC252	54M	−	C	Pre-S2
12	50141	48F	−	C	Pre-S1+2
13	46906	76M	−	C	Pre-S2
14	HCC245	44M	+	C	Pre-S1
15	60214	46M	+	C	Pre-S2

Figure 3. Schematic representation showing the status of pre-S deletion in serial samples from 15 hepatocellular carcinoma (HCC) patients with chronic hepatitis B virus (HBV) infection. Each sample is depicted as a block and its location relative to the timeline indicates the time before diagnosis of HCC. Empty blocks represent samples with no pre-S deletion, whereas shaded blocks represent samples with pre-S deletion. Demographic details of the HBV isolates at the time of diagnosis of HCC are listed at the right panel. There is no change in the pre-S deletions in all serial samples except the one labeled with an asterisk (*) where the pre-S1 deletion identified in the later samples is not found.

C, whereas 29.2% of the patients in the present study had HBV genotype B. HBV genotype C is associated with pre-S deletions and more advanced liver diseases. Sugauchi and his colleagues [11] studied the enhanced virulence of HBV genotype C compared with genotype B and hypothesized that pre-S deletions are a possible molecular mechanism for the pathogenic difference among genotypes.

Loss of pre-S1 and pre-S2 start codons were detected in HBV isolates from both HCC and non-HCC patients (Table 2). These would result in abolishment of synthesis of LHBs proteins and middle hepatitis B surface (MHBs) proteins, respectively. Though MHBs proteins may not be very important, LHBs proteins have a pivotal role in viral morphogenesis [19] and infectivity [20]. It has been suggested that pre-S wild-type HBV usually co-exists with the pre-S deletion strain so that the wild-type HBV can compensate for the defectiveness of LHB expressions in the pre-S deletion strain to sustain the viability of the viral life cycle [21].

Our study also found that deletions occurred commonly in the S promoter region (38.1%) and human serum albumin receptor region (60.0%) (Table 2). The S promoter is crucial in maintaining the balanced synthesis of the large, middle, and small form of HBsAg [22]. Dysregulation of surface gene expression may induce ER stress and hence HCC development. The functional role of human serum albumin receptor is largely unknown, and the implication of its high deletion frequency requires further studies. Mutation in the CCAAT-box may promote HBsAg retention-induced ER stress and may aggravate liver disease [23]. However, a low frequency of CCAAT-box mutation was observed in our study (16.7%; Table 2) and others [8, 11, 13]. The role of CCAAT-box in disease progression is therefore doubtful. One limitation of the present study is that we could not eliminate the potential confounding effects of precore/core promoter mutations. HBV precore/core promoter mutations have been suggested to act synergistically with pre-S deletions in the development of HCC [24, 25]. Inclusion of precore/core mutations in future studies should be performed to evaluate their interaction with pre-S deletions. To further improve the evaluation of the role of pre-S deletions for the development of HCC, clonal experiments applied to amplicons should also be considered in order to detect the existence of quasispecies as a minority of total viral population.

Although our longitudinal study cohort is limited by the number of sampling points, several findings are noteworthy. First, emergence of de novo pre-S deletions in HBV was observed in 5 (patients 1–5) of the 15 patients before the development of HCC (Figure 3). However, the possibility of harboring pre-S deletions at an earlier stage of infection in the other 10 cases cannot be excluded. Second, the time of emergence of the pre-S deletions was diverse with respect to the time of HCC development. Third, accumulation of pre-S deletions in HBV isolates was observed in 1 (patient 6) of the 15 patients, whereas no change in size and location of deletions was observed in the other patients. These findings encourage further studies to delineate the exact association between pre-S deletions and the development of HCC by serial measurements in CHB patients who eventually develop HCC.

In conclusion, our present study showed that HBV pre-S deletions, especially pre-S2 deletions, were associated with the development of HCC, irrespective of age, sex, HBeAg status, and HBV genotype. Its role in hepatocarcinogenesis may be more important in younger patients (aged <50 years) with chronic hepatitis B.

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