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ORIGINAL ARTICLE

Basic Study

Different pre-S deletion patterns and their association with hepatitis B virus genotypes

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Abstract

AIM

To investigate the associations of different types of pre-S deletions with hepatitis B virus (HBV) genotypes.

METHODS

The sequences of the pre-S region, basal core promoter (BCP) mutation, and precore (PC) mutation were examined through direct DNA sequencing or clonal analysis and sequencing in 273 HBV carriers, namely 55 asymptomatic carriers, 55 carriers with chronic hepatitis (CH), 55 with liver cirrhosis (LC), 53 with liver cirrhotic hepatocellular carcinoma (LC-HCC), and 55 with noncirrhotic HCC. A total of 126 HBV carriers (46.2%) harbored pre-S deletions. The DNA sequences of pre-S deletion mutants from 43 age-matched genotype B (HBV/B)-infected carriers and 43 age-matched genotype C (HBV/C)-infected carriers were further examined, aligned, and compared.

RESULTS

No significant difference was observed in the mean age distribution (P = 0.464), male sex (P = 0.805), viral load (P = 0.635), or BCP mutation (P = 0.117) between the HBV/B and HBV/C groups. However, the rate of PC mutation was significantly higher in the HBV/B-infected carriers than in the HBV/C-infected carriers (P = 0.003). Both genotypes exhibited a high rate of deletion in the C-terminal half of the pre-S1 region and N-terminus of the pre-S2 region (86.0% and 79.1% in the HBV/B group; 69.8% and 72.1% in the HBV/C group, respectively). Epitope mapping showed that deletion in several epitope sites was frequent in both genotypes, particularly pS1-BT and pS2-B2. Conversely, the rate of pS2-B1 deletion was significantly higher

in the HBV/B group (72.1% *vs* 37.2%, *P* = 0.002), and the rate of pS2-T deletion was significantly higher in the HBV/C group (48.8% *vs* 25.6%, *P* = 0.044). Functional mapping showed that the rate of deletion in three functional sites (the nucleocapsid binding site, start codon of M, and site for viral secretion) located in the N-terminus of the pre-S2 region was significantly higher in the HBV/B group (*P* < 0.05). One type of N-terminus pre-S1 deletion mutant with deletion of the start codon of the L protein was frequently observed in the HBV/C group (20.9% *vs* 9.3%, *P* = 0.228), particularly in the LC patients (42.9% *vs* 12.5%). Different patterns of pre-S deletions were also found between the HBV/B and HBV/C groups according to different clinical outcomes. In CH patients, deletion in the site for polymerized human serum albumin was more frequent in the HBV/B group (88.9% *vs* 36.4%, *P* = 0.028). In the LC-HCC patients, the rate of deletion in the pre-S2 region was significantly higher in the HBV/B group than in the HBV/C group (*P* < 0.05).

CONCLUSION

HBV/B- and HBV/C-infected carriers exhibit different patterns of pre-S deletion, which may be associated with the progression of liver diseases.

Key words: Hepatitis B virus; Pre-S deletion; Chronic hepatitis; Hepatocellular carcinoma; Genotype; Liver cirrhosis

Core tip: This is a comprehensive study of the influence of HBV genotypes B (HBV/B) and C (HBV/C) on the emergence of different types of pre-S deletions. Different patterns of pre-S deletion were found in HBV/B and HBV/C. Deletion in the pS2-B1 epitope, nucleocapsid binding site, start codon of M, and site for viral secretion was frequently found in HBV/B. Conversely, deletion in pS2-T and the start codon of L was frequently found in HBV/C. The prevalence of different pre-S deletions differed between HBV/B and HBV/C among patients with liver disease.

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INTRODUCTION

Hepatitis B virus (HBV) is a small, enveloped DNA virus that causes acute and chronic diseases. Most acute infections are self-limiting, whereas chronic infection may lead to the development of chronic hepatitis (CH), liver cirrhosis (LC), and hepatocellular carcinoma (HCC)[1,2]. To date, research has identified 10 HBV genotypes, designated A to J on the basis of a divergence of > 8% over the entire genomic sequence. These 10 HBV genotypes are distributed in specific geographical locations[3,4]. All genotypes can lead to progressive liver disease, but the clinical implications of each genotype differ. For example, patients infected by the genotype C (HBV/C) or D (HBV/D) strain have a higher frequency of basal core promoter (BCP) mutations, a lower response rate to interferon therapy, and more rapid progression to liver fibrosis and HCC than those infected by the genotype B (HBV/B) or A (HBV/A) strain[3,4]. In addition, carriers infected by HBV/C have a higher rate of pre-S deletions than those infected by HBV/B[5,6]. Collectively, these data suggest pathogenic and therapeutic differences among the HBV genotypes[3,4].

Three different yet structurally related HBV viral surface proteins are translated from a single open reading frame, as follows: large (L), middle (M), and small (S) proteins. The S protein consists of 226 amino acids (aa). The M protein is an extension of the S protein, with an additional 55 aa (*i.e.*, pre-S2 region). The L protein is an extension of the M protein, with an additional 108-119 aa depending on the genotype (*i.e.*, pre-S1 region). The pre-S (pre-S1 and pre-S2) region has several functional domains and plays an essential role in the viral life cycle[7-13]. The pre-S1 region can be divided into two parts: N-terminal half (aa 1-57) and C-terminal half (aa 58-119). The N-terminal half of the pre-S1 region contains the hepatocyte binding site essential for the attachment of HBV to liver cells. The C-terminal half of the pre-S1 region contains several functional sites: (1) the S promoter and the CCAAT binding factor binding site necessary for S RNA transcription; (2) the heat shock protein 70 (Hsc70) binding site and the cytosolic anchorage determinant (CAD) essential for the dual topology of L proteins; and (3) the nucleocapsid binding site (NBS) required for virion morphogenesis. The pre-S2 region contains sites for nucleocapsid binding, polymerized human serum albumin (pHSA) binding, and viral secretion (VS). The HBV pre-S region also plays an essential role in immune responses, because pre-S region carries both B-cell and T-cell epitopes[13-18]. Many studies have suggested that HBV pre-S deletions are associated with the development of progressive liver diseases[5,6,19-23]. Some in vitro studies have shown that pre-S deletion mutants can cause the accumulation of L surface proteins in the endoplasmic reticulum (ER), resulting in ER stress[22-26]. Other related studies have suggested that ER stress results in the generation of large amounts of reactive oxygen species, which can cause oxidative DNA damage, inducing mutagenesis in the genome and ultimately resulting in HCC[27-29].

Current knowledge concerning these pre-S deletion mutants is focused on the frequencies of pre-S deletion and deletion patterns in the pre-S region according to

the clinical status[5,6,19-21]. Because the pre-S region has the highest genetic variability in the whole genome, HBV genotypes may influence deletions in the pre-S region. Knowledge concerning the prevalence of different types of pre-S deletions in different HBV genotypes is limited. Therefore, this study elucidated the prevalence of different types of pre-S deletions and their associations with HBV genotypes and examined the correlation of different types of pre-S deletion with HBV genotypes according to different clinical outcomes.

MATERIALS AND METHODS

Patients

This study included 273 patients with chronic HBV infection receiving long-term follow-up at the Gastroenterology Clinic of National Taiwan University Hospital. The study population comprised 55 asymptomatic HBV carriers with a normal serum alanine aminotransferase level for at least 3 years according to periodic biochemical examinations (every 3 or 6 mo) and 218 HBsAg-positive patients with histologically verified chronic liver disease. Among the HBsAg-positive patients, 55 had CH with active viral replication, 55 had LC without HCC, 53 had liver cirrhotic HCC (LC-HCC), and 55 had noncirrhotic HCC (NC-HCC). None of them were coinfected with hepatitis C virus or hepatitis D virus. Other causes of hepatitis, including autoimmune hepatitis and alcoholic liver diseases, were excluded clinically and serologically. The serum samples of each patient were stored at -70 ℃ until use.

Hepatitis virus serologic markers

Serum HBsAg was tested using a commercial assay (Ausria-Ⅱ, Abbott Laboratories, North Chicago, IL, United States).

Extraction of serum HBV DNA and quantification of HBV DNA

Serum viral DNA was extracted using a commercial kit (QIAamp DNA Blood Mini Kit, Qiagen Inc., Valencia, CA, United States). The extracted DNA was amplified for quantifying and genotyping HBV DNA and for direct sequencing of pre-S, BCP, and precore (PC) regions, as previously described[30].

HBV DNA was quantified and genotyped as previously described[31]. The sensitivity of this real-time polymerase chain reaction (PCR) method was 10^2 copies/mL.

Determination of PC nucleotide 1896 and BCP dinucleotide 1762/1764

The segments of PC/BCP DNAs (263 bp, nucleotide positions: 1704-1966) were amplified through nested PCR and sequenced as previously described[30].

Amplification, sequencing, and cloning of HBV pre-S gene

We performed direct sequencing and clonal analysis of the pre-S region as previously described[29]. Briefly, pre-S DNA was amplified using nested PCR with two sets of HBV genotype B and genotype C-copositive primers. To avoid false-positive PCR results, precautions were strictly followed. The PCR products were electrophoresed on 2.5% agarose gel to investigate the presence of pre-S deletions. All PCR products were also directly sequenced to identify any sequence diversity or deletion.

The pre-S segments that could not be sequenced directly or those that had various small amplicons in the presence or absence of full-size amplicons (564 bp) were further investigated by clonal analysis and sequencing, as previously described[30].

Alignment

Sequence alignment was performed using the Biology WorkBench 3.2-CLUSTALW software program (http://workbench.sdsc.edu).

Ethical considerations

This study was performed in accordance with the principles of the 1975 Declaration of Helsinki and was approved by the Ethics Committees of the National Taiwan University Hospital and Fu Jen Catholic University. The sera were sampled after obtaining their written informed consent from each patient.

Statistical analysis

Data were analyzed using the Fisher exact test, χ^2 test, or contingency table with Yates' correction when appropriate. A two-sided P value of < 0.05 was considered statistically significant. All statistical analyses were performed using SPSS 12.0 for Windows (SPSS, Chicago, IL, United States).

RESULTS

Baseline characteristics of the study population

A total of 126 HBV carriers (46.2%) harbored pre-S deletions. Table 1 compares the demographic, clinical, and virological characteristics between carriers with and without pre-S deletion. No significant difference was observed in the mean age distribution ($P = 0.54$), male sex ($P = 0.054$), or PC mutation ($P = 0.132$) between them. Carriers with pre-S deletion were more frequently infected with the HBV/C strain (47.6% *vs* 28.6%, $P = 0.003$), had a higher viral load (73.0% *vs* 45.7%, $P < 0.001$), had a higher occurrence of the BCP mutation (77.8% *vs* 54.4%, $P < 0.001$), and had more progressive liver disease (CH, LC, and HCC) (96.8% *vs* 65.3 %, $P < 0.001$), particularly LC patients (28.6% *vs* 12.9%, $P = 0.001$), than those without

Table 1 Characteristics of the study population classified according to the presence of pre-S deletion *n* (%)

Features	wild type pre-S (*n* = 147)	Pre-S deletion (*n* = 126)	*P* value
Age (mean ± STD)	45.4 ± 6.6	45.9 ± 7.0	0.540
Male	101 (68.7)	100 (79.4)	0.054
Genotype C	42 (28.6)	60 (47.6)	0.003
Genotype B	94 (63.9)	62 (49.2)	
Mixed genotypes	11 (7.5)	4 (3.2)	
HBV DNA ≥ 10^5 copies/mL	67 (45.7)	92 (73.0)	< 0.001
BCP mutation (A1762T, G1764A)	80 (54.4)	98 (77.8)	< 0.001
PC mutation (G1896A)	87 (59.2)	86 (68.3)	0.132
Clinical categories			
ASC	51 (34.7)	4 (3.2)	
Progressive liver diseases	96 (65.3)	122 (96.8)	< 0.001
CH	24 (16.3)	31 (24.6)	0.098
LC	19 (12.9)	36 (28.6)	0.001
NC-HCC	29 (19.7)	24 (19.0)	1.000
LC-HCC	24 (16.3)	31 (24.6)	0.098

ASC: Asymptomatic carriers; CH: Chronic hepatitis; HBV: Hepatitis B virus; LC: Liver cirrhosis; LC-HCC: Liver cirrhotic hepatocellular carcinoma; NC-HCC: Noncirrhotic hepatocellular carcinoma; BCP: Basal core promoter; PC: Precore.

Table 2 Clinical and virological characteristics of hepatitis B virus carriers with pre-S deletion *n* (%)

Features	HBV/B (*n* = 43)	HBV/C (*n* = 43)	*P* value
Age (mean ± STD)	44.5 ± 8.8	45.8 ± 7.6	0.464
Male	33 (76.7)	31 (72.1)	0.805
HBV DNA ≥ 10^5 copies/mL	29 (67.4)	32 (74.4)	0.635
BCP mutation (A1762T, G1764A)	30 (69.8)	37 (86.0)	0.117
PC mutation (G1896A)	38 (88.4)	25 (58.1)	0.003
Clinical categories			
ASC	2 (4.7)	2 (4.7)	1.000
Progressive liver diseases	41 (95.3)	41 (95.3)	
CH	9 (20.9)	11 (25.6)	0.799
LC	8 (18.6)	14 (32.6)	0.216
NC-HCC	14 (32.6)	4 (9.3)	0.015
LC-HCC	10 (23.3)	12 (27.9)	0.805

ASC: Asymptomatic carriers; CH: Chronic hepatitis; HBV: Hepatitis B virus; LC: Liver cirrhosis; LC-HCC: Liver cirrhotic hepatocellular carcinoma; NC-HCC: Noncirrhotic hepatocellular carcinoma; BCP: Basal core promoter; PC: Precore.

pre-S deletion.

To examine the role of viral factors and to exclude the influence of HBV infection duration on the occurrence of pre-S deletion, 43 age-matched HBV/B and 43 age-matched HBV/C infected carriers were selected to examine the associations of different types of pre-S deletion with HBV genotypes. Table 2 presents the clinical and virological characteristics of 86 HBV carriers with pre-S deletion classified according to genotype. No significant difference was observed in the mean age distribution (P = 0.464), male sex (P = 0.805), viral load (P = 0.635), or BCP mutation (P = 0.117) between the HBV/B and HBV/C groups. However, the HBV/B group had a higher rate of PC mutation (88.4% *vs* 58.1%, P = 0.003) and NC-HCC (32.6% *vs* 9.3%, P = 0.003).

Table 3 Frequencies of different types of pre-S deletions in HBV/B- and HBV/C-infected carriers *n* (%)

Deletion	HBV/B (*n* = 43)	HBV/C (*n* = 43)	*P* value
N-terminal half of pre-S1 (aa 1-57)	17 (39.5)	17 (39.5)	1.000
C-terminal half of pre-S1 (aa 58-119)	37 (86.0)	30 (69.8)	0.117
N-terminus of pre-S2 (aa 1-31)	34 (79.1)	31 (72.1)	0.616
d183 M	10 (23.3)	9 (20.9)	1.000
Epitope Mapping			
Pre-S1 region			
pS1-T1 (aa 12-48 of pre-S1)	15 (34.9)	9 (20.9)	0.229
pS1-B1 (aa 12-53 of pre-S1)	15 (34.9)	9 (20.9)	0.229
pS1-B2 (aa 72-78 of pre-S1)	18 (41.9)	22 (51.2)	0.517
pS1-BT (aa 94-117 of pre-S1)	31 (72.1)	22 (51.2)	0.075
Pre-S2 region			
pS2-B1 (aa 1-6 of pre-S2)	31 (72.1)	16 (37.2)	0.002
pS2-B2 (aa 3-15 of pre-S2)	33 (76.7)	27 (62.8)	0.240
pS2-B3 (aa 13-24 of pre-S2)	19 (44.2)	24 (55.8)	0.388
pS2-T1 (aa 21-48 of pre-S2)	11 (25.6)	21 (48.8)	0.044
Functional Mapping			
Pre-S1 region (aa 1-119)			
The start codon of L	4 (9.3)	9 (20.9)	0.228
HBS (aa 2-48)	16 (37.2)	15 (34.9)	1.000
S promoter (nt 3045-3189)	31 (72.1)	30 (69.8)	1.000
CBF (nt 3137-3147)	23 (53.5)	15 (34.9)	0.128
HSC70 (aa 74-118)	31 (72.1)	29 (67.4)	0.815
CAD (aa 81-105)	30 (69.8)	23 (53.5)	0.379
Pre-S1/S2 region (aa1-174)			
NBS (aa 103-127)	39 (90.7)	27 (62.8)	0.004
Pre-S2 region (aa1-55)			
The start codon of M[1]	30 (69.8)	18 (41.9)	0.016
VS (aa 1-5 of pre-S2)	32 (74.4)	16 (37.2)	0.001
pHSA (aa 3-16 of pre-S2)	34 (79.1)	25 (58.1)	0.062

[1]Deletion or mutation of the start codon of the M protein. HBS: Hepatocyte binding site; CBF: CCAAT binding factor; HSC70: Heat shock protein 70; CAD: Cytosolic anchorage determinant; NBS: Nucleocapsid binding site; VS: Viral secretion; pHSA: Polymerized human serum albumin.

Association of different types of pre-S deletion with HBV genotypes

To investigate whether particular types of deletion in the pre-S region are associated with the HBV genotype, pre-S deletion subgenomes from 43 HBV/B and 43 HBV/C infected carriers were sequenced and aligned. The locations of these pre-S deletions are depicted in a supplementary file. All deletions were in-frame deletions with sizes ranging from 3 to 507 bp, and the deletions ended at aa 143 of the pre-S region, except for two deletions that ended at aa 150 and aa 171. Sequence alignments of pre-S deletion mutants indicated that deletion in the C-terminal half of the pre-S1 and N-terminus of pre-S2 region was more frequent in the HBV/B group (86.0% and 79.1%) than in the HBV/C group (69.8% and 72.1%), although the difference was nonsignificant (Table 3). One pre-S1

deletion mutant, d183M, which had a 183-nt in-frame deletion in the C-terminal half of the pre-S1 region, was observed in 10 of the 43 (23.3%) HBV/B-infected carriers and 9 of the 43 (20.9%) HBV/C-infected carriers (Table 3).

Epitope mapping revealed frequent deletion in two epitopes (pS1-BT and pS2-B2) in both genotypes. Conversely, deletion in pS2-B1 was significantly more frequent in the HBV/B group (72.1% *vs* 37.2%, P = 0.002), and deletion in pS2-T1 was significantly more frequent in the HBV/C group (48.8% *vs* 25.6%, P = 0.044) (Table 3).

Functional mapping revealed frequent deletion in five functional domains (S promoter, HSC70, NBS, VS, and pHSA) in the HBV/B group (rate > 70%). The rate of deletion in NBS (90.7% *vs* 62.8%, P = 0.004), the start codon of M (69.8.2% *vs* 41.9%, P = 0.016), and VS (74.4% *vs* 37.2%, P = 0.001) was significantly higher in the HBV/B group than in the HBV/C group (Table 3). Similar to HBV/B, deletion in the S-promoter, HSC70, and NBS domains was frequently observed in the HBV/C group (rate > 60%) (Table 3). Only one type of N-terminus pre-S1 deletion mutant with deletion in the start codon of the L protein was more frequent in the HBV/C group than in the HBV/B group (20.9% *vs* 9.3%, P = 0.228) (Table 3).

Correlation of different types of pre-S deletion mutant with HBV genotypes according to different clinical outcomes

The frequencies of different types of pre-S deletion mutant were compared between the HBV/B and HBV/C groups according to different clinical outcomes. The results indicated that the patterns of pre-S deletion were considerably different in the LC-HCC patients. HBV/B-infected patients had significantly higher rates of deletion in N-terminus of pre-S2 (100% *vs* 58.3%, P = 0.04), pS2-B1 (100% *vs* 25%, P < 0.001), pS2-B2 (100% *vs* 58.3%, P = 0.04), NBS (100% *vs* 50%, P = 0.015), the start codon of M (90% *vs* 25%, P = 0.004), VS (100% *vs* 25%, P < 0.001), and pHSA (100% *vs* 58.3%, P = 0.04) than did the HBV/C-infected patients (Table 4). In the asymptomatic carriers, different types of pre-S deletion seemed to correlate with the HBV genotype, but the sample size (n = 2) was too small for statistical analysis. In the CH patients, deletion in pS1-BT and four functional sites (S promoter, HSC70, CAD, and NBS), which are located in the C-terminal half of the pre-S1 region, was frequent in both genotypes. Conversely, deletion in pHSA was more frequent in the HBV/B group than in the HBV/C group (88.9% *vs* 36.4%, P = 0.028). In the LC patients, no significant differences were observed between the HBV/B and HBV/C groups, except that deletion in the start codon of L was more frequent in the HBV/C group (42.9% *vs* 12.5%, P = 0.193) (Table 4). In NC-HCC, deletion in the site for CCAAT binding factor was more frequent in the HBV/B group than in the HBV/C group (57.1% *vs* 0.0%, P = 0.092) (Table 4).

DISCUSSION

The HBV genotypes influence several aspects of HBV infection, including clinical outcomes, response to antiviral therapy, and host immune response. Pre-S deletion mutants are frequently found in patients with chronic HBV infection and are correlated with the progression of liver disease[5,6,19-23]. In the present study, the influence of the HBV genotype on the emergence of different types of pre-S deletion was examined. The results show that the frequencies of some types of pre-S deletion mutant differed between the HBV/B and HBV/C groups, whereas the frequencies of other types of deletion mutant were similar in both genotypes. Sequence alignment analysis indicated that frequent deletion in the C-terminal half of the pre-S1 and N-terminus of the pre-S2 regions was observed in both genotypes (86.0% and 79.1% in the HBV/B group; 69.8% and 72.1% in the HBV/C group, respectively). Epitope mapping of these pre-S deletion mutants showed frequent deletions in several epitope sites in both genotypes, particularly pS1-BT (72.7% in the HBV/B group and 55.8% in the HBV/C group) and pS2-B2 (75.0% in the HBV/B group and 65.1% in the HBV/C group). By contrast, the rate of pS2-B1 deletion was considerably high in the HBV/B group, and the rate of pS2-T deletion was considerably high in the HBV/C group (Table 3). Previous studies have shown that several genotype-specific antibodies are induced and react with the variable pre-S1 and pre-S2 sequences[13,14]. The host immune response to these genotype-specific epitopes may increase the selection pressure for pS2-B1 and pS2-T deletion mutants in HBV/B and HBV/C, respectively.

Functional mapping also demonstrated the similarities and differences between HBV/B and HBV/C. Deletion in the S promoter and the HSC70 site was frequently found in both genotypes. Conversely, deletion in three functional sites (NBS, the start codon of M, and VS) located in the N-terminus of the pre-S2 region was significantly more frequent in the HBV/B group (P < 0.05), and one type of N-terminus pre-S1 deletion mutant with deletion of the start codon of the L protein was frequently observed in the HBV/C group (20.9% *vs* 9.3%, P = 0.228), particularly in the LC patients (42.9% *vs* 12.5%). Previous studies in Korea have also demonstrated frequent deletion in the start codon of the L protein in HBV/C[32,33]. Unexpectedly, the tendency of deletion in functional sites is opposite between HBV/B and HBV/C. Biswas *et al*[34] similarly showed that HBV/A and HBV/C have a higher rate of pre-S2 mutation/deletion, whereas HBV/D has a higher rate of pre-S1 deletion.

The correlation of different types of pre-S deletion with the HBV genotype was further examined according to different clinical outcomes. In the CH and LC

Table 4 Frequencies of different types of pre-S deletions in HBV/B- and HBV/C-infected carriers according to their clinical outcome

Deletion	ASC		*P* value[1]	CH		*P* value[1]	LC		*P* value[1]	NC-HCC		*P* value[1]	LC-HCC		*P* value[1]
	B (*n* = 2)	C (*n* = 2)		B (*n* = 9)	C (*n* = 11)		B (*n* = 8)	C (*n* = 14)		B (*n* = 14)	C (*n* = 4)		B (*n* = 10)	C (*n* = 12)	
N-terminal half of pre-S1 (aa 1-57)	50.0%	50.0%		22.2%	27.3%		50.0%	50.0%		28.6%	50.0%		60.0%	33.3%	
C-terminal half of pre-S1 (aa 58-119)	50.0%	0		88.9%	81.8%		87.5%	71.4%		92.9%	75.0%		80.0%	66.7%	
N-terminus of pre-S2 (aa 1-31)	0	100.0%		88.9%	72.7%		62.5%	78.6%		78.6%	75.0%		100.0%	58.3%	0.040
d183 M	50.0%	0		33.3%	36.4%		25.0%	28.6%		28.6%	0		0	8.3%	
Epitope Mapping															
pS1-T1 (aa 12-48 of pre-S1)	50.0%	0		11.1%	18.2%		50.0%	28.6%		21.4%	25.0%		60.0%	16.7%	0.074
pS1-B1 (aa 12-53 of pre-S1)	50.0%	0		11.1%	18.2%		50.0%	28.6%		28.6%	25.0%		50.0%	16.7%	
pS1-B2 (aa 72-78 of pre-S1)	50.0%	0		44.4%	54.5%		37.5%	57.1%		50.0%	25.0%		30.0%	58.3%	
pS1-BT (aa 94-117 of pre-S1)	50.0%	0		88.9%	81.8%		62.5%	64.3%		71.4%	25.0%		70.0%	25.0%	0.084
pS2-B1 (aa 1-6 of pre-S2)	0	0		88.9%	45.5%	0.070	50.0%	42.9%		64.3%	50.0%		100.0%	25.0%	< 0.001
pS2-B2 (aa 3-15 of pre-S2)	0	100.0%		88.9%	54.5%		62.5%	64.3%		71.4%	75.0%		100.0%	58.3%	0.040
pS2-B3 (aa 13-24 of pre-S2)	0	100.0%		44.4%	45.5%		37.5%	64.3%		35.7%	50.0%		70.0%	50.0%	
pS2-T1 (aa 21-48 of pre-S2)	0	100.0%		33.3%	36.4%		25.0%	57.1%		14.3%	50.0%		40.0%	41.7%	
Functional mapping															
The start codon of L	0	50.0%		11.1%	9.1%		12.5%	42.9%		0	25.0%		20.0%	0	
HBS (aa 2-48)	50.0%	50.0%		22.2%	18.2%		50.0%	50.0%		21.4%	50.0%		60.0%	25.0%	
S promoter (nt 3045-3189)	50.0%	0		88.9%	81.8%		75.0%	71.4%		64.3%	75.0%		70.0%	66.7%	
CBF (nt 3137-3147)	50.0%	0		66.7%	54.5%		50.0%	42.9%		57.1%	0	0.092	40.0%	25.0%	
HSC70 (aa 74-118)	50.0%	0		88.9%	81.8%		62.5%	71.4%		71.4%	50.0%		70.0%	66.7%	
CAD (aa 81-105)	50.0%	0		88.9%	81.8%		62.5%	64.3%		64.3%	50.0%		70.0%	25.0%	0.084
NBS (aa 103-127)	50.0%	0		100.0%	90.9%		75.0%	64.3%		92.9%	50.0%		100.0%	50.0%	0.015
The start codon of M	0	0		77.8%	54.5%		50.0%	50.0%		71.4%	50.0%		90.0%	25.0%	0.004
VS (aa 1-5 of pre-S2)	0	0		88.9%	45.5%	0.070	50.0%	42.9%		71.4%	50.0%		100.0%	25.0%	< 0.001
pHSA (aa 3-16 of pre-S2)	0	100.0%		88.9%	36.4%	0.028	62.5%	64.3%		78.6%	75.0%		100.0%	58.3%	0.040

[1]$P < 0.1$. ASC: Asymptomatic carriers; CH: Chronic hepatitis; HBV: Hepatitis B virus; LC: Liver cirrhosis; LC-HCC: Liver cirrhotic hepatocellular carcinoma; NC-HCC: Noncirrhotic hepatocellular carcinoma; HBS: Hepatocyte binding site; CBF: CCAAT binding factor; HSC70: Heat shock protein 70; CAD: Cytosolic anchorage determinant; NBS: Nucleocapsid binding site; VS: Viral secretion; pHSA: Polymerized human serum albumin.

patients, frequent deletion in the C-terminal half of pre-S1 was observed in both genotypes. Significant differences were observed between the HBV/B- and HBV/C-infected patients with LC-HCC. Deletion in the N-terminus of pre-S2 region, including two epitope sites (pS2-B1 and pS2-B2) and three functional sites (the start codon of M, VS, and pHSA), was significantly more frequent in the HBV/B-infected LC-HCC patients ($P < 0.05$). In Asia, HBV/B and HBV/C commonly coexist. However, their distribution differs by country[3,4]. Previous studies have shown that pre-S2 deletion is associated with the development of HCC in Taiwan[23,35-37]. This finding may be due to HBV/B being more prevalent than HBV/C in Taiwan. HBV/C is predominant in South Korea, where studies have shown that N-terminus pre-S1 deletion mutant with deletion of the start codon of the L protein correlates with the development of HCC[32,33]. The results of the current study indicate that the tendency of different types of pre-S deletion varies across the HBV genotypes. Therefore, the difference in genotype prevalence in different countries may influence the pattern of pre-S deletion associated with progressive liver disease.

Notably, one type of N-terminus pre-S1 deletion mutant with deletion of the start codon of the L protein was frequently observed in the HBV/C group (20.9% *vs* 9.3%, $P = 0.228$), particularly in the LC patients (42.9% *vs* 12.5%). These N-terminus pre-S1 deletion mutants are similar to genotype D, which has a deletion of 11 aa in the N-terminus of the L surface protein, as reported in Korean HBV/C-infected patients[32,33]. Deletion in the start codon of the L protein may result in no synthesis of L surface proteins. Because the L protein is essential for binding to hepatocytes, formation of virion envelope, and retention of surface proteins, the absence of L

proteins not only inhibits infection and virion assembly, but also facilitates extracellular secretion of surface proteins. The intracellular retention of surface proteins of some genotype D strains has been reported in mixed infection with genotype A, which can induce hepatic carcinogenesis by activating the ER stress pathway[38]. A recent study showed that the L start codon deletion mutant resulted in the absence of L proteins and increased levels of intracellular viral mRNA and extracellular HBsAg[39]. This finding suggests that accumulated intracellular viral mRNA might activate the intracellular toll-like receptors, leading to the subsequent activation of nuclear factor kappa B pathways, chronic inflammation, and carcinogenesis[39]. The precise pathogenesis caused by these L start codon deletion mutants should be further researched in the future to determine whether it is similar to that caused by genotype D.

Previous studies have frequently detected the C-terminal half pre-S1 deletion mutant d183M in the sera of individuals with CH and cirrhosis in different countries[40-46]. This pre-S1 deletion mutant has also been found in a child with occult HBV infection[47]. In the present study, the frequency of this mutant was examined in HBV carriers with pre-S deletion. The results show that d183M was detected in 23.2% and 20.9% of the HBV/B- and HBV/C-infected carriers, respectively, and the frequency of d183M was higher in the CH patients with HBV/B (33.3%) or HBV/C (36.4%). The d183M mutant may be generated by a splicing event, because the boundary of the deletion contains consensus donor and acceptor splice sites that are conserved among all known HBV genotypes[48]. This phenomenon may explain why this unique C-terminal half pre-S1 deletion is frequently observed in patients with HBV infection. Such a deletion results in the removal of (1) the Hsc70 binding site and the CAD essential for the dual topology of L proteins; (2) the NBS required for virion morphogenesis; and (3) the S promoter necessary for S and M surface proteins. Functional characterization of this mutant revealed a defect in HBsAg secretion and viral packaging and subsequent virion secretion[26,43-46]. Western blotting and immunofluorescence analysis showed that the mutant L surface proteins are poorly secreted, heterogeneous, and accumulated within the ER[26]. Studies have demonstrated that mutant L surface proteins exhibit direct cytopathic activity when they retained in the cell[49,50]. Several clinical reports have shown that alanine aminotransferase flare-up and liver fibrosis occur following this mutation[43,44]. These clinical and functional studies strongly suggest that this mutant causes liver inflammation. Therefore, the prevalence and impact of d183M on the clinical course of infection should be investigated in a large population.

It is suggested that multiple risk factors may contribute to the pathogenesis of HBV infection. Chronic inflammation, the effect of cytokines, and the integration of HBV DNA into the host cellular genome are crucial factors in the development of HCC. In addition, HBV mutations in X, BCP, PC, and the pre-S/S region are associated with the severity of liver disease and the development of HCC[6,19-22,51,52]. The dinucleotide substitution (A1762T, G1764A) is the most common mutation in BCP. This BCP mutation is associated with the higher occurrence of HCC and LC[6,19,20,51]. Mutations in PC (G1896A) and X (C1653T and T1753V) are also associated with the development of HCC[6,51,52]. Moreover, pre-S/S mutations are associated with fulminant hepatitis, fibrosing cholestatic hepatitis, and the development of cirrhosis and HCC[6,19-22]. Recent researches suggested that microRNA is involved in HBV-related HCC[53], All of these studies indicated that both viral and host factors affect HBV pathogenesis. Additional studies should be conducted to define their role in the progression of liver disease.

To the best of our knowledge, the present study is the first comprehensive study of the influence of the HBV genotype on the emergence of different types of pre-S deletion mutant. In conclusion, some patterns of pre-S deletion differ between HBV/B and HBV/C. The prevalence of different pre-S deletion mutants differs between HBV/B and HBV/C among patients with liver disease. These differences are related to the different HBV genotypes and the different roles of pre-S1 and pre-S2 deletions in the progression of liver disease. The precise mechanisms are yet to be elucidated. Knowledge concerning HBV pre-S deletion may improve our understanding of HBV-associated hepatopathogenesis and enable establishing strategies to reduce the incidence of progressive liver diseases in patients with hepatitis infection.

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COMMENTS

Background

Chronic hepatitis B virus (HBV) infection may lead to the development of chronic hepatitis (CH), liver cirrhosis (LC), and hepatocellular carcinoma (HCC). To date, research has identified 10 HBV genotypes, designated A to J. All genotypes can lead to progressive liver disease, but the clinical impaction of each genotype differs. For example, patients infected by the genotype C (HBV/C) strain have a higher frequency of basal core promoter (BCP) mutations, a lower response rate to interferon therapy, and more rapid progression to liver fibrosis and HCC than those infected by the genotype B (HBV/B) strain.

Research frontiers

Naturally occurring HBV pre-S deletion mutants have been identified and are associated with the development of progressive liver disease in hepatitis B carriers. Because the pre-S region has the highest genetic variability in the whole genome, the HBV genotypes may influence the deletions in the pre-S region. Knowledge concerning the prevalence of different types of pre-S

deletions in different HBV genotypes is limited.

Innovations and breakthroughs

This study is the first comprehensive study to investigate the associations of different types of pre-S deletion with HBV genotypes. The results reveal different patterns of pre-S deletion between HBV/B- and HBV/C-infected carriers. This finding may be attributable to the progression of liver disease.

Applications

These findings show that the different patterns of pre-S deletion mutants are associated with the HBV genotype. Different types of pre-S deletion are also found between HBV/B- and HBV/C-infected patients with different types of liver disease. Knowledge concerning HBV pre-S deletion may improve our understanding of HBV-associated hepatopathogenesis and enable establishing strategies to reduce the incidence of progressive liver diseases in patients with hepatitis infection.

Peer-review

Author of this manuscript described the differential patterns of pre-S deletions in association with HBV genotypes. This is an interesting and innovative work. It is very useful information to predict the prognosis and clinical outcomes. It would be better to compare/describe the pre-S/S mutations including pre-S deletions, too.

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