Original Article

Hepatitis B Virus S Promoter Deletion in Hepatocellular Carcinoma

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Objective To identify the difference and significance of dominant types of hepatitis B virus (HBV) pre-S mutation between liver tumor tissues and paired adjacent non-tumor tissues and to test if the mutations were tumor tissue specific.

Methods HBV DNA isolated from 34 paired intratumoral and peritumoral tissues of hepatocellular carcinoma (HCC) patients were screened by PCR and direct sequencing. All patients carried HBV with genotype C, except for one B/C heterozygote. The expression, localization and excretion of LHBs mutant carrying pre-S deletions were characterized in vitro. The expression of endoplasmic reticulum (ER) GRP78 mRNA was assayed.

Results Four patterns of pre-S mutations were identified: pre-S1 in-frame deletion involving the first start codon; pre-S2 in-frame deletion; pre-S2 start codon mutation with or without in-frame deletion; and S promoter in-frame deletion (Δ SP). The first two types were evenly found in both tumor and non-tumor tissues. They were rarely present as dominant strains. The last two types were frequently found in the dominant strains in tumor tissues. The overall prevalence of HBV carrying Δ SP was 17.64% (6/34) in tumor tissues, but none were dominant in non-tumor tissues. HBV carrying Δ SP was unable to produce S protein in vitro. Immunocytofluorescence assay showed that the Δ SP LHBs mutant aggregated in the cytoplasm, accumulating mainly in the ER. Transient transfection and expression of Δ SP mutant caused GRP78 up-regulation in vitro.

Conclusions HBV S promoter deletion was found dominantly in HCC tumor tissue. The aggregation of mutant large surface proteins in the ER possibly involved in HBV-related HCC.

Key words: Hepatitis B virus; S promoter deletion; ER stress; Hepatocellular carcinoma

Hepatitis B virus (HBV) is a hepatotropic virus that causes acute and chronic liver diseases. China is an intermediate endemic area with HBsAg prevalence of 7.18% in the general population,¹ where the chronic HBV infection is believed to be usually acquired prenatally, or in early childhood.² Chronic HBV infection is recognized as an important risk factor for hepatocellular carcinoma (HCC).³ It has been estimated that more than 90% of HCC is etiologically associated with HBV in China.⁴

HBV contains four open reading frames (ORFs), encoding the surface proteins, the core antigen and e antigen, the polymerase and the HBx protein. The surface antigens are encoded by the pre-S/S (pre-surface/surface) region of the genome containing a single S-ORF with three in-frame start codons which are responsible for large (L), medium (M) and small (S) surface proteins. The pre-S/S region is transcriptionally

controlled by two separate promoters, pre-S1 and S promoter. Pre-S1 drives 2.4kb mRNA encoding L, M and S surface proteins, while S drives 2.1kb mRNA encoding only M and S surface proteins.

The highest rates of mutations in the HBV genome can be found in the pre-S1 and pre-S2 regions where point mutations, deletions or genetic recombinations occur.⁵⁻⁸ Our previous studies and recent reports suggested that a pre-S deletion correlated with disease progression, and that these pre-S deletions were an independent risk factor for HCC.⁵⁻⁸

Deletions in the S promoter region (nt 3045-3180) completely or partially removed the binding sites for transcription factors such as NF-1 and SP1.⁹ Thus, it affects the expression of large, middle and small surface proteins, resulting in intracellular accumulation of mutant large surface proteins.¹⁰⁻¹² The transcriptional regulatory activity of pre-S mutant surface protein has been proposed.¹³ The accumulation of unfolded or misfolded proteins in the endoplasmic reticulum (ER) lumen induces ER stress which has been suggested to play an important role in hepatocyte damage and regeneration, and therefore be involved in

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hepatocarcinogenesis.^{11,14,15}

It was reported that the prevalence rate of serum HBV pre-S deletions was increased during the disease progression from asymptomatic carrier (ASC) to end stage liver diseases like cirrhosis or HCC.^{8,12,16-19} Accumulating evidences in recent years have revealed that some mutant viral proteins exhibit increased oncogenic prosperity.^{4,15,20} However, one may argue that the increased detection frequency of HBV DNA mutations may simply reflect the over time mutation accumulation, and the selection of host immune suppression.^{17,21-24}

In this study, we compared different types of HBV pre-S region mutations in paired tumor and non-tumor tissues to confirm if there were specific types of pre-S mutations that were dominant in tumor tissues, and furthermore, to elucidate the direct oncogenic evidence of pre-S mutation.

MATERIALS AND METHODS

Patients

A total of 34 HCC patients (HBsAg positive) were recruited from the Henan Cancer Hospital (the patient's age ranged from 30 years to 70 years; mean = 50.94 ± 8.00 years). None of the patients had undergone interferon therapy or antiviral treatment. All tissue samples were collected from 2006 to 2008 and stored at -70°C. All cases of HCC diagnosed were confirmed at the corresponding hospital using the criteria set by the European Association for the Study of the Liver (EASL).²⁵ The pathological diagnosis of HCC and corresponding non-tumor tissue was determined according to the WHO classification. The patients enrolled in this study fulfilled the following criteria: hepatitis B surface antigen (HBsAg)-positive by enzyme immunoassay (EIA, Abbott Laboratories, Abbott Park, IL, USA); all were negative for antibodies against hepatitis C virus (HCV) (EIA, Abbott Laboratories); autoimmune liver disease, drug-related hepatitis, alcoholic hepatitis and obstructive jaundice were excluded. This study was approved by the Ethics Committee of Peking University Health Science Center, and an informed consent was obtained from each patient.

Sample preparation and detection of pre-S mutations

Genomic DNA was extracted from snap frozen HCC tumor tissues and corresponding non-tumor liver tissues using proteinase K followed by the standard phenol/chloroform extraction and ethanol precipitation method.

The pre-S region was amplified by nested Polymerase chain reaction (PCR). External primers were SL1 (5'-TTC TTG GGA ACA AGA TCT ACA GC-3', nt 2803-2822) and SL2 (5'-CCC CAA CTT CCA ATT

ACA TAT CC-3', nt 902-880). Internal primers were P1 (5'-TCA CCA TAT TCT TGG GAA CAA GA-3', nt 2823-2845) and S4R (5'-AGA AGA TGA GGC ATA GCA GC-3', nt 436-417).

The expected second round PCR product is roughly 800 base pairs (bp), containing the whole pre-S gene and partial S region. PCR was carried out in a volume of 50 µl containing 0.1 µg template, 5 µl 10 × buffer, 1 µl 10 mmol/L dNTP, 2 µl 10 µmol/L sense and antisense primers and 0.5U Taq DNA polymerase (BGI Life Tech Co., Ltd, China). The first round of PCR was performed under the following conditions: 95°C for 5 min, followed by 40 cycles of 95°C for 20 s, 57°C for 20 s and 72°C for 90 s, and a final extension at 72°C for 7 min. The second round of PCR was performed with 2 µl of the first round PCR products as templates under the following conditions: 95°C for 5 min, followed by 95°C for 20 s, 55°C for 20 s and 72°C 1 min for 40 cycles, and a final extension at 72°C for 7 min. The second round PCR products were visualized on a 1% agarose gel. All necessary precautions to prevent cross-contamination were taken. Two HCC tissues from patients' negative of HBV infection sera markers and 1 blank control were included in each assay. PCR products were purified using gel purification kit (Axygen, USA) and sequenced (ABI Prism 3700 DNA analyzer, USA).

Sequence analysis and genotyping

Nucleotide sequences were compared with the HBV DNA sequences of wild-type genotype "C" registered in GenBank (NCBI), AY123424.1. The nucleotide sequences were determined by the NCBI HBV Genotyping Tool (<http://www.ncbi.nlm.nih.gov/projects/genotyping/formpage.cgi>).

In vitro expression and secretion of the cloned pre-S mutants in Huh-7 cell line

Site-directed mutagenesis and plasmid construction

The HBV DNA sequence (2423-3215/1-1969) was amplified from pZAC-1.2×HBV plasmid (Genotype C, GenBank AY123424.1). The PCR product was inserted into plasmid pGL3-Basic which lacks eukaryotic promoter and enhancer sequences, named as pGL3-preS/Swt. Meanwhile, pGL3-preS/SΔsp with nt 3020-3202 deletion (GenBank AY123424.1) was constructed. All constructs were confirmed by sequencing.

Cell lines and transfection

The human hepatoma cell lines HepG2 and Huh-7 were used for *in vitro* cell culture studies. The cells were maintained in regular Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (Gibco BRL, GrandIsland, NY). The cells were grown at 37°C with 5% CO₂. Plasmid DNA transfection was carried out by the liposome induction method (Lipofectamine 2000, Invitrogen, USA) according to the

manufacturer's instruction.

Detection of HBV surface proteins in culture supernatants and cell lysate of transfected cells

The HepG2 cells were transiently transfected with pGL3-preS/Swt, pGL3-preS/SΔsp, and pGL3-Basic plasmid. The supernatants were collected 48 hours after transfection and cell debris in the medium was removed by centrifugation. Quantitative HBsAg was studied by automated chemiluminescent microparticle immunoassay (Architect HBsAg, Abbott, IL). Cells were lysed through repeated freezing and thawing in RIPA lysate buffer (Beyotime, Beijing) and the lysates were harvested. Total of 60 µg total proteins were resolved on SDS-polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes. The membrane was incubated with the antibody conjugated with horseradish peroxidase. The proteins were detected by using ECL chemiluminescence kit (Promega, USA). The antibody used for Western blot was the polyclonal antibody against all three HBV surface antigens including the large, middle and small HBsAg (Keyuezhongkai, Beijing).

Indirect immunofluorescence using confocal laser scanning microscopy

After 48 hours post-transfection, the Huh-7 cells were processed for detection of endoplasmic reticulum by using ER tracker Red DPX (Invitrogen, USA) labeling by immunofluorescence according to the manufacturer. Meanwhile, the cells were immuno-stained with goat anti-HBs antibody for 1 hour at 37°C. After washing with PBST for 5 times, the slides were then incubated with secondary anti-goat antibody conjugated with fluorescein isothiocyanate (FITC). Finally, the cell nuclei were counterstained with Hoechst 33342. The sections were visualized by confocal microscopy at a wavelength of 547 nm for ER tracker Red DPX, 480 nm for FITC and 345 nm for Hoechst 33342. Confocal laser scanning microscopy was performed by a Zeiss LSM 410 microscope, and images were processed with the Adobe Photoshop 3.0.5 program.

Quantitative analysis of ER chaperon GRP78 via realtime RT-PCR

After 36 hours post-transfection, total RNA was extracted from cells transiently transfected with the pGL3-Basic (Basic), pGL3-preS/Swt, and pGL3-preS/SΔsp plasmid separately using Trizol reagent (Invitrogen, USA) according to the manufacturer's protocol. Then, the RNA was treated with DNase I (NEB, USA). And cDNA was reversely transcribed from 4.0 µg of total RNA with random hexamer primers using an MMLV-reverse transcriptase kit (Fermentas, MBI) as recommended by the supplier.

qRT-PCR was performed on the LightCycler 480 real-time PCR system (Roche, German) using the QuantiTect SYBR Green PCR kit (Roche, German). The 20 µl reaction mix contained 200 nmol/L of each primer, 10 µl of LightCycler 480 SYBR green I master

mix (Roche), and 1 µl of template cDNA. The primers for the grp78 gene were F (5'-GCC TGT ATT TCT AGA CCT GCC-3') and R (5'-TTC ATC TTG CCA GCC AGT TG-3'), and those used for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene were F (5'-AGA AGG CTG GGG CTC ATT TG-3') and R (5'-AGG GGC CAT CCA CAG TCT TC-3').

For the GRP78 and GAPDH, the amplifications consisted of 10 min at 95°C followed by 35 cycles, each consisting of denaturing for 10 s at 95°C, annealing for 10 s at 60°C, and elongation for 10 s at 72°C. Amplifications were performed in triplicate in LightCycler 480 real-time PCR instrument. Negative controls containing non human cDNA template were included for each gene within each PCR run. Amplification specificity for each gene was confirmed by a single distinct melting curve. PCR products were separated using 1.5% agarose gel electrophoresis to confirm the presence of a single band at the expected amplicon size.

Statistical analysis

All statistical analyses were performed using the Statistical Analysis System (SAS 8.2). Associations in 2 × 2 tables were evaluated with Fisher's exact test. The comparison between categorical variables was tested by the chi-square test. All tests were 2-sided and a *P* value less than 0.05 was considered to be statistically significant.

RESULTS

Demographical and clinical data of HCC patients

The demographic, disease and virological clinical data of 34 patients with HCC are given in Table 1a and 1b. The mean serum alanine transaminase (ALT) level was 49.25 ± 20.30 U/ml, and the percentage of positive anti-HBe was 60.61% (20/33) in these patients. Tissue DNAs from 25 paired HCC tumor and non-tumor samples and 9 HCC tumor samples were amplified for HBV pre-S/S region. Phylogenetic analysis and subgenotype-specific analysis revealed that among 34 HBV-infected HCC patients, the proportions of genotype C2 and C2/Ba were 97.06% (33/34) and 2.94% (1/34), respectively. The HBV C2/Ba strains were detected in patient with HCC-11 tumor tissue.

Detection and location of pre-S mutation in tumor and non-tumor tissue

Four types of pre-S region mutations were found: pre-S1 start codon in-frame deletion (ranging from the first amino acid to the 11st amino acid) (type I), pre-S2 in-frame deletion (type II), pre-S2 start codon mutation with or without pre-S2 in-frame deletion (type III), and S promoter region in-frame deletion (type IV) (Figure 1, Figure 2).

The distribution of pre-S mutations in tumor and non-

tumor liver tissue was illustrated in Table 1. The 4 types of pre-S deletions were all in-frame deletions according to our sequencing data. The overall percentages of pre-S mutation were 44.12% (15/34) in tumor tissue and 48.00% (12/25) in non-tumor tissue separately.

The rates of all the mutant types did not differ

significantly between tumor tissue and non-tumor tissue ($P > 0.05$) (Table 1). However, the frequency of type II mutation was higher in non-tumor group as compared to tumor group (40.00% (10/25) vs. 20.59% (7/34) ($P = 0.104$). The type IV mutation strains were found to be the dominant population in 17.64% (6/34)

Table 1a. The demographic, disease and virological characteristics of HCC patients with coupled tissues

Sample	Sex	Age (year)	HBeAb	ALT (U/ml)	Pre-S1 deletions	Pre-S2 start codon	Pre-S2 deletions
HCC-3	F	66	–	26	Tumor: W/O Non-tumor: 1st strain: W/O; 2nd strain: nt 2846-2863	W/O	W/O
HCC-6	M	46	–	85	Tumor: 1st strain: W/O; 2nd strain: nt 2850-2864	W/O	W/O
HCC-5	M	58	+	29	Tumor: W/O Non-tumor: 1st strain: W/O; 2nd strain: nt 3040-3081	W/O	Tumor: 1st strain: W/O; 2nd strain: nt 25-54 Non-tumor: W/O
HCC-16	M	52	–	80	Tumor: 1st strain: W/O; 2nd strain: nt 2847-2867 Non-tumor: W/O	W/O	Tumor: 1st strain: W/O; 2nd strain: 5-55; Non-tumor: W/O
HCC-8	M	41	–	39	W/O	W/O	Tumor: W/O; Non-tumor: 1st strain: W/O 2nd strain: nt 10-54
HCC-10	M	52	+	24	Tumor: W/O Non-tumor: 1st strain: W/O; 2nd strain: nt 2835-2852	W/O	Tumor: 1st strain: W/O; 2nd strain: nt 18-53; 3rd strain: nt 39-53 Non-tumor: 1st strain: W/O; 2nd strain: nt 18-53
HCC-12	M	46	–	42	Tumor: W/O Non-tumor: 1st strain: W/O; 2nd strain: nt 2847-2864	W/O	Tumor: 1st strain: W/O; 2nd strain: nt 34-57 Non-tumor: 1st strain: W/O; 2nd strain: nt 34-57
HCC-13	M	45	+	58	W/O	W/O	Tumor: W/O non-tumor: 1st strain: W/O; 2nd strain: nt 17-55
HCC-14	M	49	–	52	W/O	W/O	Tumor: 1st strain: W/O; 2nd strain: nt 49-56 non-tumor: 1st strain: W/O; 2nd strain: nt 49-56
HCC-26	M	69	+	132	W/O	W/O	Tumor: W/O Non-tumor: 1st strain: W/O; 2nd strain: nt 25-54
HCC-29	M	45	–	25	W/O	W/O	Tumor: W/O Non-tumor: 1st strain: W/O; 2nd strain: nt 37-54
HCC-28	M	61	–	96	W/O	W/O	Tumor and non-tumor: 1st strain: W/O; 2nd strain: 19-54
HCC-4	M	70	–	NA	Tumor: W/O Non-tumor: W/O	WT	Tumor: nt 3206-3215, 1-5 Non-tumor: W/O
HCC-15	F	43	–	85	W/O	Tumor and non-tumor: 1st strain: nt 3204-3215, 1-9, 28-54; 2nd strain: W/O	
HCC-2	M	30	+	42	Tumor: 1st strain: nt 2860-2988 & 3019-3080; 2nd strain: 2860-2988 & 3019-3080, 6 bp insertion at nt15; 3rd strain: W/O Non-tumor: W/O	Tumor: W/O Non-tumor: ATR	W/O
HCC-11	M	54	+	51	Tumor: solely strain: nt 2852-2884 & 2894-3080 Non-tumor: W/O	W/O	W/O
HCC-24	M	62	+	45	Tumor: solely strain: nt 3020-3202 Non-tumor: W/O	W/O	W/O
HCC-25	M	36	–	NA	Tumor: 1st strain: W/O; 2nd strain: nt 2991-3073 Non-tumor: W/O	W/O	Tumor: W/O Non-tumor: 1st strain: W/O; 2nd strain: nt 25-54
HCC-9	M		–	NA	W/O	W/O	W/O
HCC-18	F	42	+	13	W/O	W/O	W/O
HCC-19	M	56	–	65	W/O	W/O	W/O
HCC-21	M	44	+	42	W/O	W/O	W/O
HCC-22	M	55	+	35	W/O	W/O	W/O
HCC-23	M	40	–	37	W/O	W/O	W/O
HCC-27	M	50	+	22	W/O	W/O	W/O

Table 1b. The demographic, disease and virological characteristics of 9 HCC patients with only tumor tissues

Sample	Sex	Age (year)	Anti-HBe	ALT (U/L)	Pre-S1 deletions	Start codon	Pre-S2 deletions
HCC-7	M	68	+	56	W/O	ACG	nt 45-59
HCC-36	M	42	+	39	1st stain: nt 2958-3068; 2nd strain: W/O; 3rd strain: nt 2817-2846	WT	W/O
HCC-38	M	49	+	58	Tumor: 1st strain:W/O; 2nd strain: nt 2877-3083	WT	W/O
HCC-1	M	55	+	42	W/O	WT	W/O
HCC-35	M	61	+	20	W/O	WT	W/O
HCC-37	M	53	+	24	W/O	WT	W/O
HCC-39	M	43	+	43	W/O	WT	W/O
HCC-41	M	50	-	62	W/O	WT	W/O
HCC-42	M	42	+	85	W/O	WT	W/O

Notes: ALT denotes alanine aminotransferase, Cut-off ≤ 40 IU/L; W/O denotes without

Table 2. Distribution of pre-S mutations in the tumor and non-tumor tissue

Sample	No. (%) with mutation ^a	No. (%) of cases with mutations			
		Type I	Type II	Type III	Type IV
Tumor	44.12% (15/34)	8.82% (3/34)	20.59% (7/34)	11.76% (4/34)	17.64% (6/34)
Non-tumor	48.00% (12/25)	12.00% (3/25)	40.00% (10/25)	8.00% (2/25)	4.00% (1/25)
P value	0.767	1.000	0.104	1.000	0.232

Note: ^aindicates the sample containing multiple mutant strains which were counted once

tumor tissues. In contrast, only 1 of 25 non-tumor tissues was positive for the mutation, and it was present non-dominantly.

Mutant and wild type HBV strains coexisted in most of the samples tested. It is important to notice that viruses harboring type I and II mutations were not dominant in viral population (Table 2). In contrast, the type III and IV mutant strains were dominant in tumor tissues. Moreover, the type IV mutant in tumor tissues was present as the dominant or sole strain in viral population according to the DNA electropherogram.

Aberrant excretion of the HBV surface protein with S promoter deletion mutation

The amount of surface protein in the culture supernatant of the HepG2 cells transiently transfected with the pGL3-Basic, pGL3-preS/Swt, and pGL3-preS/SΔsp (ΔSP) were tested by automated chemiluminescent microparticle immunoassay at 12 h and 48 h after transfection.

The background value of HepG2 cells transfected with the pGL3-Basic vector alone was adjusted to zero during automated chemiluminescent microparticle immunoassay of the culture supernatant. The reduced secretion of HBV surface protein was detected in HepG2 cells transfected with ΔSP mutant constructs compared with wild-type at 48 h after transfection (Figure 3).

S promoter deletion mutation affects S protein production and causes a change in the large vs. small surface protein ratio

The 39-kDa unglycosylated (p39) and 42-kDa glycosylated (gp42) forms of the L protein were found

in the lysate of cells transiently transfected with wild-type (WT) expression vector. The truncated LHBs migrated faster than the wild-type LHBs and had a higher expression level compared to that of wild-type LHBs. However, the 24-kDa unglycosylated (p24) and 27-kDa glycosylated (gp27) forms of the small surface protein were only detected in the lysate of cells transfected with wild-type expression vector only (Figure 4). In contrast, the small surface protein was undetectable in HepG2 transfected with pGL3-pre-S/SΔsp.

Effect of S promoter deletion mutation affects HBV surface proteins subcellular distribution

The Huh-7 cells were transiently transfected with pGL3-preS/Swt, and pGL3-preS/S Δsp, labeled by ER tracker Red DPX, and stained for HBsAg by immunofluorescence at 48 h after transfection. The cells were visualized by confocal microscopy. The following observations were made: HBV surface proteins were diffused in cytoplasm, ER tracker colocalized in the Huh-7 cells transfected with WT plasmid. The mutant large surface protein aggregated in the endoplasmic reticulum in Huh-7 cells transfected with ΔSP plasmid. The mutant large surface protein exhibited the same subcellular localization pattern as the normal large surface protein (Figure 5).

ER stress signal gene GRP78 induced by the S promoter mutants

The protein encoded by GRP78 is localized in the lumen of the endoplasmic reticulum (ER), and is involved in the folding and assembly of proteins in the ER. The expression of GRP78 and GAPDH was detected

Figure 1. The illustration for different types of pre-S mutants.

Notes: Type I : Pre-S1 start codon in-frame deletion resulted in first 11aa deleted LHBs; Type II : Pre-S2 in-frame deletion; Type III: Pre-S2 start codon mutation with or without in-frame deletion abolished M proteins; Type IV : S promoter in-frame deletion abolished M/S proteins

Figure 2. The DNA electropherogram of pre-S amplicon with pre-S type III and type IV mutations.

Notes: The arrows indicate the site deletion mapped. The black arrow indicated the start site of pre-S deletion. The red arrow showed the start site of pre-S2 start codon deletion. The black box showed the pre-S2 start condon deletion.

Figure 3. Detection of surface protein excretion in culture supernatant from the HepG2 cells transfected with the pGL3-Basic, pGL3-preS/Swt, and pGL3-preS/Δsp.

Notes: HepG2 cells were transiently transfected with various plasmids at 12 h and 48 h post-transfection. They were wild-type (WT) and S Promoter deletion (ΔSP). The data were from three independent experiments.

Figure 4. Western blot analysis of the HBsAg in cell lysates from the transiently transfected HepG2 cells.

Notes: HepG2 cells were transfected with HBV S promoter wild-type (WT) plasmid and S promoter deletion (ΔSP) vectors. A total of 60μg of Proteins were extracted from HepG2 cells 48h post-transfection and intracellular viral proteins. HBsAg (large surface protein and small surface protein) were detected with an anti-HBs antibody. Anti-α-tubulin staining of the same blot was performed to prove equal loading. The plasmids which expressed large surface protein and small surface protein (generously provided by Professor Wenhui Li, NIBS, China) were cotransfected as positive control which showed in line 3.

in cells transiently transfected with the pGL3-Basic, pGL3-preS/Swt and pGL3-preS/Δsp for 48 h. As compared with pGL3-Basic, the WT showed slightly enhanced expression of GRP78 (average = 1.46 folds) while the ΔSP mutant showed an obvious, enhanced expression level of GRP78 (average = 6.44 folds) (Figure 6).

DISCUSSION

Previous studies by our lab and others showed that HCC patients with chronic HBV infection had a higher

prevalence of HBV with pre-S deletions than chronic HBV infected patients without HCC.^{6,8,26,27} Indeed, HBV pre-S2 deletions have been suggested as an independent factor associated with the development of HCC.²⁶ However, the observed association was essentially based on cross-sectional or case control studies. Nevertheless, almost all of such studies used serum from the patients as specimens. We still need more direct evidence to link HBV pre-S deletions with hepatocarcinogenesis and in vitro oncogenic characteristics studies of pre-S mutations, in order to determine their roles at the molecular mechanistic level.

Figure 5. Subcellular localization of HBs detected by immunofluorescence analysis in the HuH-7 cells transfected with pGL3-pre-S/Swt (WT), and pGL3-pre-S/S Δsp (ΔSP).

Notes: The cells were stained with ER tracker Red DPX (red) to mark proteins in the endoplasmic reticulum. HuH-7 cells tranfected with WT and ΔSP mutant were stained with goat anti-HBS. Anti-goat antibody conjugated with FITC (green) and ER tracker red DPX (red) were then simultaneously incubated with the cells (a wavelength of 547 nm for ER tracker Red DPX, 480 nm for FITC and 345 nm for Hoechst 33342). The images of HBS were merged with ER tracker staining to show their localizations.

Panel 1: WT-subcellular location of HBV surface protein transfected with wild-type plasmid; Panel 2: L-subcellular location of large surface protein only; Panel 3: ΔSP-subcellular location of HBV surface protein transfected with s promoter deletion plasmid

Figure 6. Dectecion of GRP78 relative expression in cells transfected transiently with the pGL3-pre-S/Swt, and pGL3-pre-S/S Δsp.

Notes: Transcriptional activation of ER stress genes GRP78 by ΔSP mutant was observed. The GRP78 expression levels were normalized with GAPDH expression. There is a one fold ratio of GPR78 vs.GAPDH expression in cells transfected with pGL3-Basic vector.

In this study, we analyzed HBV pre-S mutations in paired tumor and the corresponding adjacent non-tumor tissue specimens.

Firstly, our data indicated that all deletions in pre-S1/S2 region were in-frame deletions. Thus the predicted pre-S1 and/or pre-S2 proteins contained internal deletions, and that the integrity of polymerase ORF was maintained.

Secondly, we found that the pre-S1 start codon in-frame deletion coexisted with the wild-type strain and various mutation strains in both tumor and the non-tumor tissues. The type I mutation of HBV pre-S region (pre-S1 start codon in-frame deletion) permits synthesis of the large surface protein from the second in-frame methionine codon, resulting in the deletion of the first 11 amino acids of LHBs. The prevalence of such mutations had no difference between tumor and non-tumor specimens studied ($P = 1.000$). Omission of the first 11 amino acids of LHBs is a distinct characteristic of HBV genotype D. However, nearly all patients studied here were infected with HBV genotype C.

Thirdly, HBV with pre-S type II mutation (pre-S2 region contains epitopes for T and B cells, i.e. HLA-3 restricted T-cell epitopes (amino acids 109 to 123), B-cell epitopes for neutralizing (amino acids 120 to 145)).^{17,22,23,28,29} Thus, viruses harboring such mutations will lose CTL epitopes and become dominant in virus population under the persistent pressure of host's immune selection. As expected, since HBV strains harboring such mutations promote continued infection and persistent inflammation, we thought this sort of mutations may still play an indirect role in HBV related hepatocarcinogenesis.

Fourthly, the HBV type III mutation (pre-S2 start codon mutation with or without pre-S2 in-frame mutation) was frequently found in serum of HCC patients, and has been suggested as an independent factor associated with the development of HCC.^{8,15,21,26,27} A study from this laboratory had demonstrated that HBV harboring pre-S2 nt4-57 deletion accompanied by pre-S2 start codon mutation led to a dramatic decrease in the synthesis of small-sized and middle-sized surface antigens. Furthermore, they showed that pre-S2 LHBs mutant could directly interact with the Jun activation domain-binding protein (JAB1), and induce hyperphosphorylation of tumor suppressor retinoblastoma (RB) via cyclin-dependent kinase 2 (cdk2).³⁰ Therefore, the pre-S2 mutant LHBs was suggested to be a potential oncoprotein for HBV-related HCC. However, this kind of mutation was found in dominant strains in only 8.82% (3/34) of tumor and 4% (1/25) of non-tumor tissues in the present study.

Finally and interestingly, type IV mutation (HBV with S promoter in-frame deletion) was found in the dominant strains in 6 of 34 tumor tissues. Such mutation was found in only one non-dominant strain in one non-tumor tissue. The dominant strain was judged

based on the DNA sequencing electropherogram. The discovery of such mutation as dominant strain solely in tumor tissue, implicated a direct involvement of HBV with S promoter in-frame deletion in hepatotumorigenesis. Nevertheless, such mutations may be either from the subcellular relax circular DNA or from the integration of HBV carrying the type IV mutation.³¹

The pre-S1 region contains the S promoter (nt 3045-3180) which regulates the production of small surface protein. Pre-S2/S promoter mutant in this region will lead to a deletion of some or all 4 SP1 binding sites (3066-3085, 3123-3142, 3146-3167, 3158-3182) (the numbering is with reference to genotype C, GenBank AY123424.1) and NF-Y binding site, i.e.CCAAT (3137-3141).⁹ The deletions in our study occurred from nucleotide 2860-2988 & 3020-3094, 2852-2884 & 2894-3080, 3020-3202, 3197-3215, 2958-3068, and 2877-3083 in HCC-2, HCC-11, HCC-24, HCC-25, HCC-36 and HCC-38 tumor samples, respectively. The mutations we found partially or totally overlapped at the above sites. Therefore, HBV carrying such mutations were expected to exhibit a reduced production of small surface protein. In the present study, the secretion of surface protein was undetectable in culture supernatants of HepG2 cell line transiently transfected with Δ SP plasmid, which was significantly different from the high expression of surface antigen with the HepG2 cells transfected with wild-type HBV construct. In addition, we found that in HepG2 cells transiently transfected with the S promoter mutant construct, the expressed surface antigen was dominated by LHBs. In contrast, the production of middle and small surface protein could not be detected (Figure 4). Except for the LHBs' excretion deficiency, immunocytofluorescence further showed that unlike the whole cell diffusion of wild type LHBs, the Δ SP LHBs mutant showed an aggregated cytoplasmic pattern, and was found to mainly accumulate in ER (Figure 5). A heat shock protein 70 (Hsc70) is involved to maintain the dual topology of the pre-S region of the L protein and proper intracellular transportation.^{32,33} The pre-S1 region nt 3038-3170 (aa 63 to 107 of LHBs) contains the interaction site(s) for Hsc70 binding site. The deletions we found here were all patially mapped to the Hsc70 binding site. Therefore, mutant LHBs lacking Hsc70 could cause cytoplasmic aggregation. An alternative explanation for the cytoplasmic aggregation could be due to the lack of S surface protein, since the S surface protein is necessary for the L protein's proper intracellular transportation.³⁴

It has been reported that the LHBs had several potential HLA-restricted B- and T-cell epitopes in pre-S1 region.^{17,22-24,29} Therefore, HBV harboring the type IV mutation possesses an advantage under the host immune pressure, which favors infection persistence. On the other hand, the retention of LHBs with S

promoter deletion in ER can induce unfolded protein response and activate ER stress.^{14,35} As expected, our experiment in vitro revealed that the excessive mutant protein caused up-regulation of GRP78 mRNA expression. GRP78 over-expression can up-regulate important survival signals and was involved in a variety of human malignancies.³⁶⁻⁴³

Furthermore, the ER stress initiated by pre-S mutant LHBs could induce oxidative DNA damage and genomic instability.⁴⁴

In summary, our study reveals that pre-S mutations, especially pre-S2 deletions are relatively common in liver tumor and non-tumor tissues. HBV mutations of S promoter in-frame deletion were found to be dominated in tumor tissues only. This type IV mutation abolished both M and S surface protein synthesis, caused excessive production of mutant LHBs, and thus induced ER stress due to its improper intracellular transportation and ER accumulation. The improper ER retention of mutant LHBs induced ER stress and the subsequent events are believed to be the underlying mechanism for the direct tumorigenic property of type IV pre-S mutation.

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