Journal of Digestive Diseases and Hepatology
Zhu P, et al. J Dig Dis Hepatol 6: 182.
www.doi.org/10.29011/2574-3511.100082
www.gavinpublishers.com

OPEN ACCESS

GAVIN PUBLISHERS

Research Article

Analysis of Hepatitis B Virus Pre-S Gene in Hepatocellular Carcinoma and Paracancerous Tissues from Southern China

Ping an Zhu[1,2*], Peiyao Shi[1], Leijing Duan[1], Xian Jin[1], Yang Tan[1], Fenfang Lei[2], Qingxia Liu[3]

[1]Department of Experimental Diagnosis, The Yantianqu People's Hospital of Shenzhen, Guangdong, Shenzhen, China

[2]Shaoyang University, Hunan, Shaoyang, China

[3]Department of Experimental Diagnosis, Xiangya Hospital, Central South University, Hunan, Changsha, China

[*]**Corresponding author:** Ping an Zhu, Department of Experimental Diagnosis, The Yantianqu People's Hospital of Shenzhen, Guangdong, Shenzhen 518081, China

Citation: Zhu P, Shi P, Duan L, Jin X, Tan Y, et al. (2022) Analysis of Hepatitis B Virus Pre-S Gene in Hepatocellular Carcinoma and Paracancerous Tissues from Southern China. J Dig Dis Hepatol 6: 182. DOI: https://doi.org/10.29011/2574-3511.100082

Received Date: 30 August, 2022; **Accepted Date:** 12 September, 2022; **Published Date:** 15 September, 2022

Abstract:

Chronic hepatitis B virus (HBV) infection is one of the major causes of hepatocellular carcinoma (HCC), and the HBV pre-S gene plays a critical role in the molecular pathogenesis of HBV-related HCC. We have investigated whether there are particular HBV pre-S gene mutations associated with HCC in patients from southern China. The HBV pre-S gene was examined in 100 paraffin-embedded tumor tissue and 100 paraffin-embedded paracancerous tissue samples from patients with HCC by nested polymerase chain reaction (PCR). The HBV pre-S genes with potentially important mutations from tumor tissue samples were sequenced and aligned with the published HBV pre-S gene sequence. Twelve patients (12.0%) had pre-S mutations; six had a pre-S1 deletion, one patient had a pre-S2_ deletion, five patients had both a pre-S deletion, and a pre-S2 mutation. Among them, two patients contained pre-S1 insertion and two patients contained pre-S1 start codon mutation. Two patients (2.0%) of hepatocellular carcinoma paracancerous tissue had pre-S mutation and two patients had pre-S1 + pre-S2 deletion. The pre-S sequence of HBV contained stop codon in four patients of HCC, leading to truncated pre-S protein and changed protein structure. These results suggest that HBV pre-S gene deletion mutation and insertion mutation in HCC tissues and paracancerous tissues lead to truncated HBV pre-S protein or protein changes, which may be related to the occurrence of hepatocellular carcinoma.

Keywords: Hepatocellular carcinoma; Paracancerous tissue; Hepatitis B virus; Pre-S gene; Mutation

Introduction

Hepatitis B virus (HBV) infection is a major threat to global public health; which can lead to many acute and chronic liver diseases. Chronic hepatitis B virus infection is a major risk factor for hepatocellular carcinoma (HCC). HCC is the leading cause of cancer-related death in the world and the fourth most common malignant tumor and the second leading cause of cancer-related death in China [1-3].The HBV virus is a DNA virus belongs to the Hepadnaviridae family. The HBV-DNA is a 3.2kb circular; partial double strand genome. All coding information is on the minus DNA strand and it is organized into four open reading frames. Despite hepatitis B virus is a DNA virus; it has a high mutation rate due to its replicative strategy that leads to the production of many non-identical variants at each cycle of replication [4].

The S region of HBV consists of Pre-S1 gene; pre-S2 gene and S gene. HBV S gene encodes a major surface protein (HBsAg). HBV PreS1 gene (nt.2848-3204) and pre-S2 gene (nt.3205-154) overlap with P gene and encode pre-S1 and pre-S2 protein. The pre-S region is located upstream of the s-open reading frame of

HBV genome; which is crucial to the life cycle of HBV. In the natural course of HBV infection; the accumulation of naturally occurring mutations in the HBV genome can generate several mutant types of HBV-encoded proteins; including three different proteins in the S ORF (SHBs; MHBs; and LHBs) and HBcAg in the C ORF; which could contribute to enhanced ER stress in infected hepatocytes mainly via increased ER accumulation of mutant proteins. Endoplasmic reticulum (ER) stress induction by HBV infection has been implicated in liver carcinogenesis and disease progression with chronic inflammation via enhanced inflammation; oxidative stress-mediated DNA damage; and hepatocyte proliferation [5].In order to further explore the role of hepatitis B virus pre-S mutant in the pathogenesis of hepatocellular carcinoma in China; this study analyzed the pre-S gene in hepatocellular carcinoma and paracancerous tissues (HCCP).

Material and Methods

Specimens

Primary hepatoma samples of 100 patients (88 males and 12 females; mean age 51 years; range 25-77 years) were collected from the Affiliated Xiangya Hospital of Central South University (Changsha; China) in 2016 and 2017.7hese patients were natives and permanent residents of different places of non-HCC-prevalent area in southern China such as Hunan; Hubei and Jiangxi provinces; which were diagnosed by liver biopsy or the combination of increased a fetoprotein (>25 ng/ml) and typical features on angiography; sonography or computed tomography. None of these patients had previously received HBV vaccine. The control group comprised 100 paraffin-embedded paracancerous tissue samples from the same patients with HCC. All serum samples were tested for HBsAg; hepatitis B surface anti-body (anti-HBs); hepatitis B e antigen (HBeAg); hepatitis Be antibody (anti-HBe) and hepatitis B core antibody (anti-HBc) using commercially available kits (Abbott Laboratories; North Chicago; USA). Serum alpha-fetoprotein levels were measured by hemiluminescence immunoassay. All patients were tested negative for antibody to hepatitis C virus and positive for HBV surface antigen. Tumor tissues were collected after surgical resection; fixed in formalin and preserved in paraffin sections. The sera of HBV carriers were collected separately and stored at -80 °C until used.

Preparation of DNA for PCR analysis

Paraffin-embedded HCC tissues and Paracancerous tissues DNA extraction

Genomic DNAs from the above mentioned 100 patients were isolated from the paraffin-embedded HCC tissues and paracancerous tissues as described and with some modifications [6]. Briefly; an approximate 100 mg of HCC tissues was dewaxed with xylene and cleaned with anhydrous ethanol. The samples were homogenized in 1.5 ml lysis buffer (50 mM Tris-HCl; oH 7.5; 150 mM NaCl; 2 mM EDTA) and incubated overnight with 0.4 mg/ml proteinase K and 1% SDS at 37°C. After extraction with phenol:chloroform (1:1;viv); the pooled aqueous phase was transferred to a fresh centrifuge tube and followed by an addition of 0.2 volume of 3 M sodium acetate (pH 7.0) and two volume of ethanol and precipitated overnight at -20°C. The DNA pellet was washed with 70% cold ethanol and resuspended in 50 ulof 10 mM Tris-HCl; pH 7.4; and 1 mM EDTA. Serum DNAs were extracted using HBV DNA PCR-Fluorescence Quantitative Diagnostic Kit (SKHB; Shanghai; China). The concentration of DNA was determined by spectrophotometry at 260 nm. The purity was determined by calculating the ratio of absorbance at 260 nm to absorbance at 280 nm. The purified DNA was diluted to a final concentration of 500 ng/ul.

Serum DNA extraction

According to the kit instructions; the operation steps are as follows: take 100 u .The concentrated solution was added into a 1.5 ml centrifuge tube; and then 100 ml of serum was added ul. Shake and mix for 5 s; 12 000 rom for 5 min; and discard the supernatant. Join 20 u After shaking for 5 s; centrifugation for several seconds; constant temperature treatment at 100 °C for 10 min; centrifugation at 12 000 R/ min for 5 min; and supernatant 2 uL was used as template for PCR amplification.

Controls for PCR. Negative controls were performed using DNA samples extracted from liver tissues of healthy donors without any HBV markers; PCR buffer without DNA; or water only. Positive controls were: HepG2.2.15; containing the full length of the HBV gene.

Nested PCR

The HBV genomic fragment was amplified by a two-step nested PCR with four primers specific to the coding region of HBV pre-S. These primers include: P1: 5 - TGCGGGTCACCATATTCTTG-3 (nt.2811-2830; outer sense); P2: 5 - CCGCCTGTAACACGAGCA-3 (nt.206-189; outer antisense); 03: 5 - TTGGGAACAAGATCTACA -3 (nt.2828-2845; inner sense) ;P4: 5 - GGTCCTAGGAGTCCTGATGC -3 (nt.186-166; inner antisense): The first and second PCR were performed using Pfu DNA polymerase for 35 cycles. The first-round PCR was amplified with primer1 and primer2 and performed in 25u1 volume containing 2x MasterMix (10mM Tris-HCl; pH 8.3; 50mM KCI; 1.5mM MgCl2; 0.25mM of each dNTP (TIANGEN; Beijing; China); 0.5uM of primer P1 and P2; 0.1U/u1Pfu DNA polymerase (TIANGEN; Beijing; China) and 1u1 template DNA. Thermocyler condition was 5min at 94°C; 35cycles of 30s at 94°C; 30s at 52°C; 60 s at 72°C; and a final extension at 72°C for 5 min. The resulting PCR product was used as a template DNA for second-round amplification. The second-round PCR was amplified with primer3 and primer4; amplification thermocycling was 5 min

at 94°C; 35 cycles of 30s at 94°C; 30 s at 52°C; 60 sat 72°C and a final extension of 72°C for 5 min. The first-round PCR was to amplify a DNA fragment of 611bp; and the second-round PCR products were to 574bp.

Gene sequencing

Nested PCR was amplified by HBV primers; PCR products were electrophoretic on 2.5% agarose gel; PCR products were sent to Shanghai bioengineering biotechnology Service Co.; Ltd.; ABI PRISM 3730 DNA sequencer (Applied Biosystems; Foster City; and Biosystems) were sequenced; and the software was used for comparison and analysis to determine any sequence diversity or deletion.

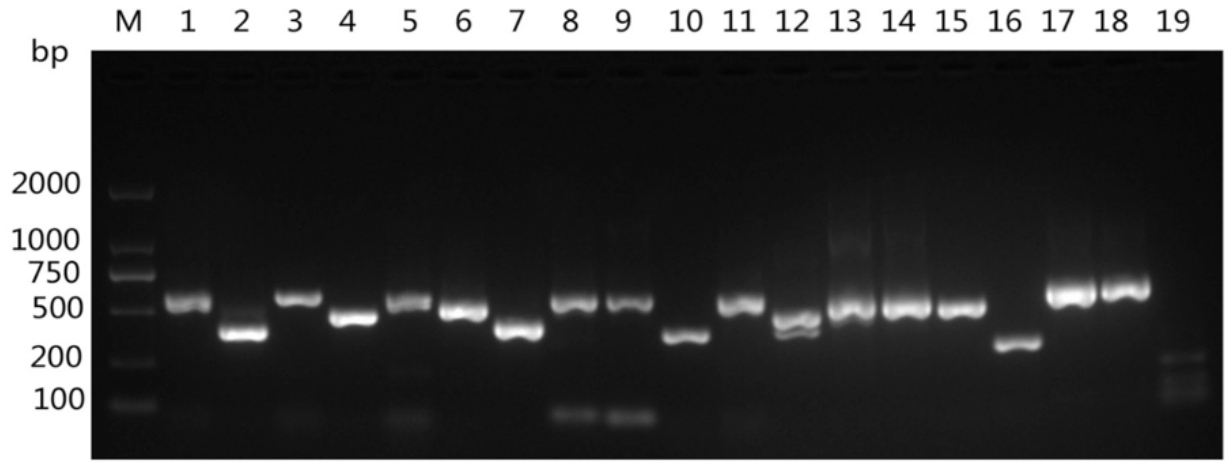
HBV genotyping

HBV pre-S gene was analyzed by genotyping tools (http:/ Avwww.ncbi.nim.nih.gov/ projects/genotyping/formpage.cgi) to in out gene sequence of HCC tissues or paracancerous tissues. HBV pre-S gene was compared with reference sequence for HBV genotype analysis.

Results

PCR amplification of HBV pre-S gene

HBV preS gene of HCC tissues; paracancerous tissues; positive control and negative control DNA were nested PCR; the second round PCR amplification product of HBV gene were used by 2.5% agarose gel electrophoresis. The detection rate of HBV pre-S gene was 44% in 100 HCC tissues; 43% in paracancerous tissues; and 31% in HCC tissue S and paracancerous tissues. The electrophoretic map of some PCR products is shown in Figure 1.



M: DNA size marker (D2000); Lane1:HCC1; Lane 2:HCC14; Lane 3: HCCP14; Lane 4: HCC27; Lane 5: HCC35; Lane 6: HCC60; Lane 7: HCC64; Lane 8:HCCP64; Lane 9:HCC73; Lane 10: HCCP76; Lane11:HCC82; Lane 12:HCC83; Lane13:HCCP85; Lane 14:HCC95; Lane 15: HCC97; Lane 16:HCC99; Lane1/7-18: positive control for the PCR amplification; Lane19: negative control for the PCR amplification.

Figure 1: Amplification of HBV preS gene by PCR with primers P3 and P4.

Sequencing and genotyping of hepatitis B virus pre-S gene in hepatocellular carcinoma and paracancerous tissue

HBV pre-S gene was imported into HCC tissues and paracancerous tissues by gene sequencing and genotyping tools; and compared with the reference sequence for HBV genotyping analysis. 22 HCC tissues were sequenced positive; including 5 genotype C and 17 genotype B (22.73% (5 / 22) and 77.27% (17 / 22); respectively; Among the 17 paracancerous tissues positive samples; 2 were genotype C and 15 were genotype B; accounting for 11.76% (2/17) and 88.24% (15 / 17); respectively.

Sequencing of hepatitis B virus pre-S gene in hepatocellular carcinoma

In 100 cases of HCC; 20 cases were positive for HBV pre-S gene sequencing; and the target gene was compared with the reference sequence of hepatitis B virus (AY518556). 10 cases of HCC (HCC1; HCC27; HCC35; HCC60; HCC73; HCC82; HCC83; HCC95; HCC97; and HCC99) were lack of pre-S gene; and the consistency of gene and amino acid sequence was 41.95% - 88.70%; respectively 13.22% ~ 85.63%. There was one deletion mutation in 6 samples; and there were two stop codons in HBV

pre-S60 sequence; There were 2 deletion fragments in 2 samples; and 7 stop codons in HBV pre-S35 sequence; There was one deletion + insertion mutation in two samples. HBV pre-S73 contained five stop codons. The including a stop codon of HBV pre-S genes will lead to early termination of translation; resulting in truncated HBV pre-S protein and altered protein structure. There were point mutations in the HBV pre-S gene in the other 10 HCC tissues; and the homologies of HBV pre-S gene and amino acid sequences were 76.25% - 99.43% and 67.24% - 98.28%; respectively.

In addition; the pre-S1 gene (nt.2848-3204) and pre-S2 gene (nt.3205-154) of HBV pre-S1; HBV pre-S2; and pre-S1 + pre-S2 gene ranges from H BV nt2848-3204; HBV; HBV nt3205-154; and HBV nt2848-154; respectively. HBV pre-S deletion mutants in hepatocellular carcinoma tissues included pre-S1; pre-S2 and pre-S1 + pre-S2 mutants. Among them; pre-S1 mutants (HCC35; HCC60; HCC73; HCC82; and HCC99) accounted for 50.00% (5/10); pre-S2 mutants (HCC1) accounted for 10.00% (1/10); and pre-S1+ pre-S2 mutants (HCC27; HCC83; HCC95; HCC97) accounted for 40.00% (4 / 10) (Table 1).

Number	HBV pre-S Gene	Target Gene	Deletion Site and Mutant	Insertion Position and Gene	Alignment Consistency with Reference Sequence (%)		HBV Genotype
					Gene	Amino acid	
1	HBV pre-S1	489bp	HBV nt.24-57; 33bp; pre-S2 mutant	/	81.14%	78.74%	C
2	HBV pre-S27	417bp	HBV nt.3124-3215;1-14; 105bp; pre-S1+pre-S2 mutant	/	78.35%	75.86%	B
3	HBV pre-S35	410bp	HBV nt.2848-2879; 31bp; HBVnt.2905-2986;82 bp; pre-S1 mutant	/	63.86%	13.22%	C
4	HBV pre-S60	471bp	HBV nt.3119-3170; 51bp; pre-S1 mutant	/	88.70%	85.63%	B
5	HBV pre-S73	520bp	HBV nt.2957-2972; 16bp; pre-S1 mutant	HBV nt.3089-3 104; 15bp	67.04%	14.37%	C
6	HBV pre-S82	474bp	HBV nt.3158-3205; 48b p; pre-S1 mutant	A(HBV nt.3124)	65.84%	43.10%	C
7	HBV pre-S83	417bp	HBV nt.3121-3215; 1-10; 105bp; pre-S1+pre-S 2 mutant	/	77.20%	75.86%	B
8	HBV pre-S95	465bp	HBV nt.3202-3207; 6bp; HBVnt.3-53; 51bp; pre-S1+pre-S2 mutant	/	86.59%	82.76%	B
9	HBV pre-S97	438bp	HBV nt.3147-3215; 1-15; 84bp; pre-S1+pre-S2 mutant	/	82.76%	81.03%	B
10	HBV pre-S99	240bp	HBV nt.2851-3132; 282bp; pre-S1 mutant	/	41.95%	41.95%	B

Table 1: Comparison of HBV pre-S gene or amino acid in hepatocellular carcinoma tissues with wild-type HBV pre-S gene or amino acid reference sequence (AY518556)

Sequencing of hepatitis B virus pre-S gene in hepatocellular carcinoma and paracancerous tissues

After gene sequencing; 6 samples (HCC8; HCCP8; HCC14; HCCP14; HCC26; HCCP26; HCC52; HCCP52; HCC64; HCCP64; HCC91; and HCCP91) were simultaneously positive for HBV pre-S gene in 100 HCC tissues and adjacent tissues. Compared with the reference sequence (AY5185564); the deletion position and fragment of HCC14 and HCC64 were inconsistent; including pre-S1; pre-S1 + pre-S2 mutants; there were point mutations in other HCC tissues and paracancerous tissues. The sequence identity of gene and amino acid in HCC tissues was 64.37% ~ 99.23% and 63.22% ~ 98.85% respectively. The sequence identity of gene and amino acid in paracancerous tissues was 87.93% ~ 99.43% and 83.91% ~ 98.85% respectively. HBV pre-S64 has two deletion fragments; including a stop codon in the sequence; which leads to the truncated HBV pre-S protein and the change of protein structure (Table 2, Figure 2).

Number	HBV pre-S Gene	Target Gene	Deletion Site and Mutant	Insertion Position and Gene	Alignment Consistency with Reference Sequence (%)		HBV Genotype
					Gene	Amino acid	
1	HBV pre-S8 (HCC)	522bp	/	/	98.85%	97.70%	B
2	HBV pre-S8 (HCCP)	523bp	/	C (HBV nt.114)	98.28%	89.66%	B
3	HBV pre-S14 (HCC)	339bp	HBV nt.3020-3202; 183bp; pre-S1 mutant	/	64.37%	63.22%	B
4	HBV pre-S14 (HCCP)	522bp	/	/	96.74%	93.10%	B
5	HBV pre-S26 (HCC)	522bp	/	/	87.93%	85.06%	C
6	HBV pre-S26 (HCCP)	522bp	/	/	87.93%	83.91%	C
7	HBV pre-S52 (HCC)	522bp	/	/	99.23%	98.28%	B
8	HBV pre-S52 (HCCP)	522bp	/	/	98.66%	96.55%	B
9	HBV pre-S64 (HCC)	369bp	HBV nt.2981-3108; 127bp; HBV nt.3188-3214; 26bp; pre-S1+pre-S2 mutant	/	70.11%	61.49%	B
10	HBV pre-S64 (HCCP)	522bp	/	/	99.43%	98.85%	B
11	HBV pre-S91 (HCC)	522bp	/	/	98.66%	97.70%	B
12	HBV pre-S91 (HCCP)	522bp	/	/	99.23%	98.28%	B

Table 2: Comparison of HBV pre-S gene from hepatocellular carcinoma and adjacent tissues and pre-S gene among wild-type HBV genotype B (AY518556).

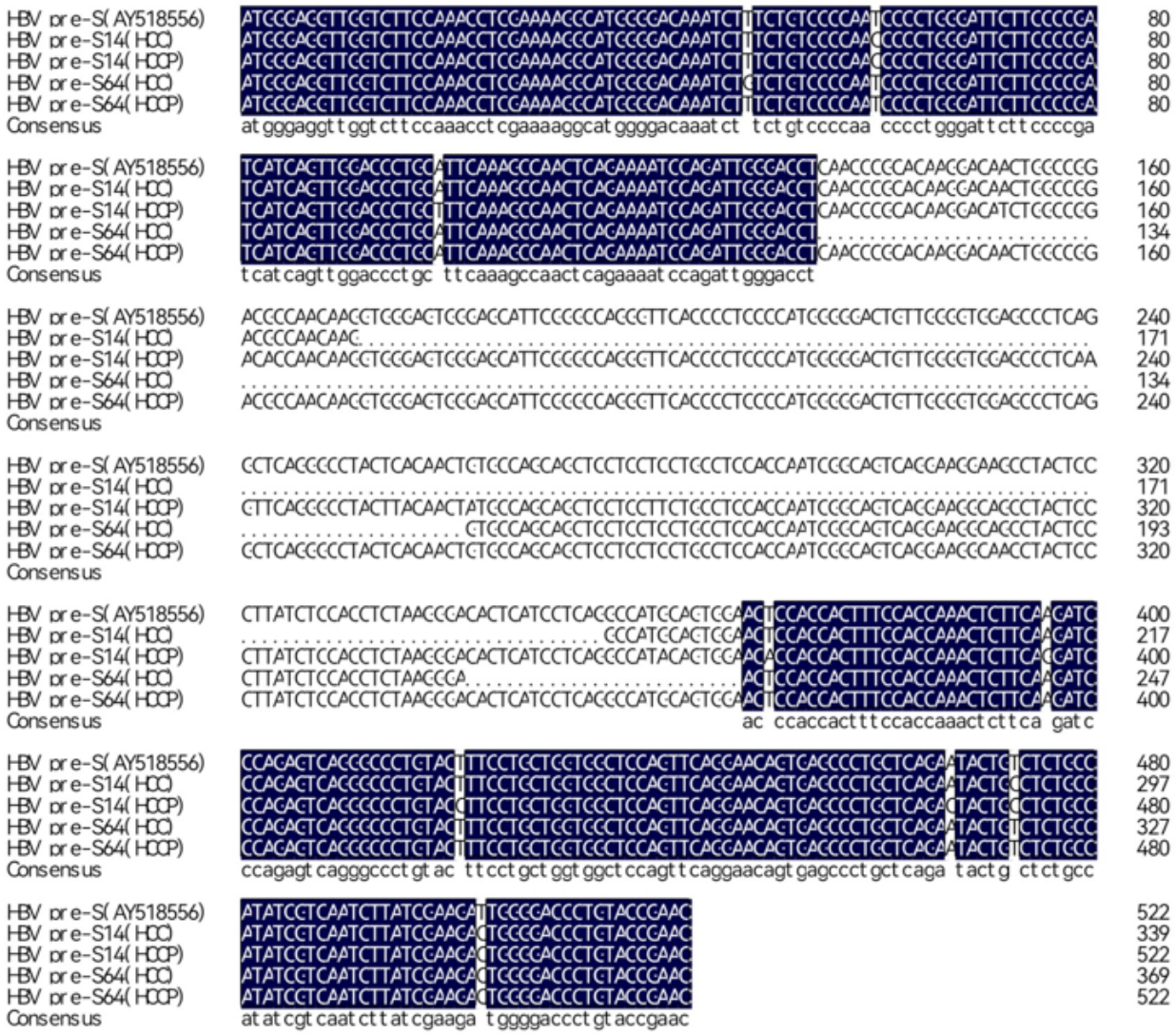


Figure 2: Comparison of HBV pre-S gene from hepatocellular carcinoma and paracancerous tissues and pre-S gene among wild-type HBV genotype B (AY518556).

Sequencing of hepatitis B virus pre-S gene in paracancerous tissues of hepatocellular carcinoma

Seventeen of 100 HCC paracancerous tissues were positive for HBV pre-S gene sequencing; of which 2 (HCCP76; HCCP85) samples were HBV pre-S gene deletion; and 15 samples were HBV pre-S gene point mutation. Compared with the reference sequence (AY518556); HBV pre-S76 had two deletion fragments; the deletion position and fragment were HBV nt.2938-3105168bp and HBV nt.3206-3215; 1-23;33bp; respectively. There were pre-S1+pre-S2 mutants in HCCP76 and HCCP85; and the homology of gene and amino acid sequence was 59.2% ~ 99.62% and 55.17% ~ 98.85% respectively. In addition; there were 17 paracancerous tissues of HCC; of which 15 samples were HBV genotype B and 2 samples were HBV genotype C. HCCP/76 and HCCP85 were type B (Figure 3).

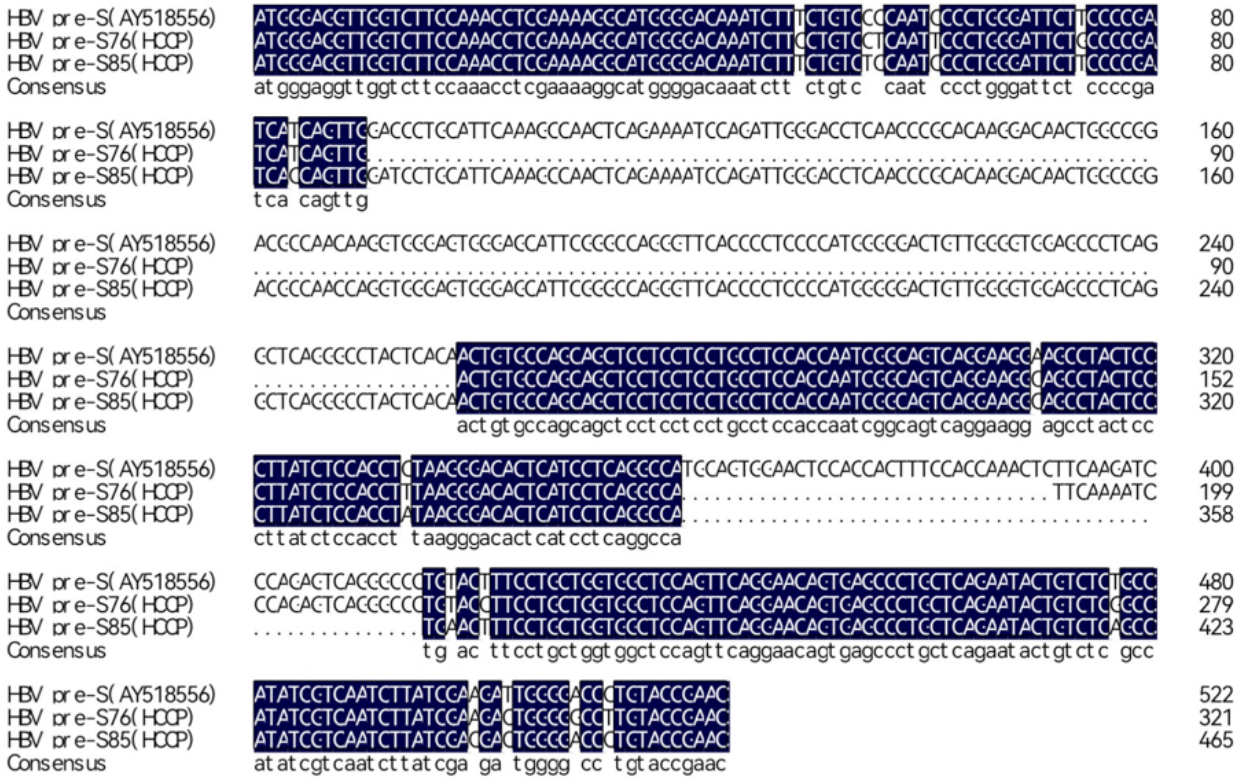


Figure 3: Comparison of HBV pre-S gene from paracancerous tissues of hepatocellular carcinoma and pre-S gene among wild-type HBV genotype B (AY518556).

Discussion

Hepatitis B virus (HBV) contributes to hepatocellular carcinoma (HCC) development through direct and indirect mechanisms. HBV DNA integration into the host genome occurs at early steps of clonal tumor expansion and induces both genomic instability and direct insertional mutagenesis of diverse cancer-related genes. Prolonged expression of the viral regulatory protein HBx and/or altered versions of the pre-S/S envelope proteins dysregulates cell transcription and proliferation control and sensitizes liver cells to carcinogenic factors. Accumulation of preS1 large envelope proteins and/or preS2/S mutant proteins activates the unfold proteins response; that can contribute to hepatocyte transformation [7].

In this study; 10 HBV pre-S gene deletion fragments were found; including pre-S1 mutant (50.00%); pre-S2 mutant (10.00%); pre-S1 + pre-S2 mutant (40.00%); as shown in Table 1.Yeung et al.[8] were found that Pre-S deletions were determined in HBV isolates from 115 chronic hepatitis B (chronic hepatitis B; CHB) patients with HCC. HBV pre-S deletions were clustered mainly in the 3' end of pre-S1 and 5 end of pre-S2 regions. Patients with HCC had a higher prevalence of HBV with pre-S deletions than did patients without HCC. In particular; only pre-S2 deletions but not pre-S1 deletions were significantly associated with the development of HCC. 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. HBV pre-S2 deletions were an independent factor associated with the development of HCC. Pre-S/S variants are often identified in hepatitis B carriers with CH; LC; and HCC. According to the mutation type; five pre-S/S variants have been identified: pre-S deletion; pre-S point mutation; pre-S1 splice variant; C-terminus S point mutation; and pre-S/S nonsense mutation. Different pre-S/S variants cause liver diseases through different mechanisms. Most cause the intracellular retention of HBV envelope proteins and induction of endoplasmic reticulum stress; which results in liver diseases [9]. The pre-S1 and pre-S2 mutant large HBV surface antigen (LHBS); in which the pre-S1 and pre-S2 regions of the LHBS gene are partially deleted; are highly associated with HBV-related HCC The pre-S deletion rates were relatively low (7%) in the sera of patients with acute HBV infection. They gradually increased in periods of persistent HBV infection: pre-S mutation rates were 37% in chronic HBV carriers; and as high as 60% in HCC patients [10].Hepatitis B virus (HBV) pre-S mutants; which harbor deletions over pre-S1

and pre-S2 gene segments of large surface proteins; have been implicated in HCC recurrence. We demonstrated that the presence of deletions spanning the pre-S2 gene segment and the high percentage of pre-S2 plus pre-S1 + pre-S2 deletions; either alone or in combination; was significantly and independently associated with poor recurrence-free survival and had greater prognostic performance than other clinicopathological and viral factors in predicting HCC recurrencel11].Wang et al. have identified two types of large HBV surface antigens (LHBs) with deletions at the pre-S1 (DeltaS1-LHBs) and pre-S2 (DeltaS2-LHBs) regions in ground glass hepatocytes. The pre-S mutant LHBs are retained in the endoplasmic reticulum (ER) and escape from immune attack. The pre-S mutants; particularly DeltaS2-LHBs; are increasingly prevalent in patients with hepatitis B e antigen (HBeAg)-positive chronic HBV infection. In HCC patients; the two pre-S mutants were detected in 60% of HCC patients; in the serum and in HCC tissues. Pre-S mutant LHBs can initiate ER stress to induce oxidative DNA damage and genomic instability. Furthermore; pre-S mutant LHBs can upregulate cyclooxygenase-2 and cyclin A to induce cell cycle progression and proliferation of hepatocytes. In the present study; 64 patients with HBV-related hepatocellular carcinoma (HCC) were categorized into two groups according to the presence or absence of pre-S mutation(s). Nineteen patients (29.7%) had pre-S mutations; 13 had a pre-S deletion; three had a pre-S2 start codon mutation; two patients had both a pre-S deletion; and a pre-S2 start codon mutation; and one patient had a pre-S2 insertion [13] The deleted regions were clustered mainly in the C terminus of pre-S1 (70.5%) and the N terminus of pre-S2 (72.7%) in HCC patients. Immuno-epitope mapping of these pre-S deletion sequences showed that all the deletion regions encompassed I- and B- cell epitopes and the B-cell epitope at amino acid 1-6 of pre-S2 was significantly deleted in HCC patients (60.0% vs. 0.0%:P = 0.036). Deletion of B-cell epitope at amino acid 1-6 of pre-S2 region and the site for virion secretion are significantly associated with the development of HCC in HBV carriers [14].Zhang et al.[15] were found that Thirty-two chronic hepatitis B (CHB) patients with HCC (HCC group) and 32 matched CHB patients without HCC (controls) were recruited. Compared with control patients; HCC patients had a significant greater quasispecies complexity; greater diversity; and a trend of greater complexity at the amino acid level. HCC patients had a higher intrapatient prevalence of pre-S deletions and point mutations (at codons 4; 27; and 167) compared with the control patients. Longitudinal observation in the sera of 14 HCC patients showed that quasispecies complexity and diversity increased as the disease progressed to HCC. Ground glass hepatocytes identified in HBV-related hepatocellular carcinoma (HCC) patients harbor pre-S deletion variants that largely accumulate in the ER lumen due to mutation-induced protein misfolding and are associated with increased risks of cancer recurrence and metastasis[16].PreS1 deletion was most frequently detected in LC patients while preS2 deletion was most frequently detected in HCC patients; both frequencies were significantly higher than that in CHB patients (17.1% vs. 4.8%; P<0.01; 19.1% vs. 4.8%; P<0.01). The deletion patterns across preS gene were different among the 4 illness categories. Compared with wild-type strain; the preS1 deletion mutant had defected preS1 expression; significantly decreased viral MRNA level and SP II promoter activity; while preS2 deletion mutant had defected preS2 expression; and significantly decreased viral mRNA level. HBV preS deletion was associated with advancement of liver diseases not only presented in preS deletion incidence; but also in the deletion pattern. Patients with preS2 deletion might have a higher risk to develop HCC [17]. Pre-S deleted proteins are naturally occurring mutant forms of HBV large surface proteins that are expressed by HBV surface genes harboring deletion mutations over the pre-S gene segments. It has been well demonstrated that HBV pre-S deleted proteins function as important oncoproteins; which promote malignant phenotypes of hepatocytes through the activation of multiple oncogenic signaling pathways and result in HCC formation. The oncogenic signaling pathways activated by pre-S deleted proteins have been verified as potential therapeutic targets for the prevention of HCC development. Moreover; the presence of pre-S gene deletions and the expression of pre-S deleted proteins in the blood and liver tissues of HBV-infected patients have been evaluated as valuable biomarkers for predicting a higher risk of HCC development and recurrence after curative surgical resection [18].

There were 6 positive samples of HBV pre-S gene sequencing in HCC tissues and paracancerous tissues. There was one deletion in HBV pre-S14 and two deletions in HBV pre-S64 in HCC tissues. There was one stop codon in HBV pre-S64 sequence. There was a point mutation in other HCC tissues and paracancerous tissues. In addition; two samples were found to have HBV pre-S gene deletion. There were two HBV pre-S/6 deletion fragments; and pre-S1 + pre-S2 mutants in HCCP76 and HCCP85. Hepatitis B virus DNA was extracted from the tumor and non-tumor tissues of 16 HCC patients. Overlapping DNA fragments covering the entire HBV genome were amplified and sequenced. Fourteen patients had full-length HBV amplification. Hot-soot mutations at HBx aa130-131 and pre-S deletions were detected in 13 (93%) and 6 (43%) patients; respectively. Deletions in the X/preC/C regions were more frequently detected in the tumor than in the non-tumor tissues. Compared with the non-tumor tissues; the tumor tissues had a lower quasispecies complexity (P = 0.014 and 0.043; at the nucleotide and amino acid levels; respectively) and diversity (P = 0.048 and 0.022; at the nucleotide and amino acid levels; respectively). Phylogenetic analysis showed that HBV sequences derived from tumor and non-tumor tissues were separately clustered; suggesting the occurrence of compartmentalization; which was confirmed by the correlation coefficient testing on both the number and length of branches of viral populations. Hepatitis

B virus mutation patterns in HCC tumor tissues and non-tumor tissues were different. HBV quasispecies within the pre-S region were compartmentalized; and tumor tissues had a lower genome complexity and diversity [19].

22 cases were positive for HCC tissue sequencing; 22/3% of C genotype and 77.27% of B genotype; The C genotype was 11./6% and B genotype was 88.24%. There are pre-S1 mutants; pre-S2 mutants and pre-S1 + pre-S2 mutants; as shown in Table 1 and Table 2. HBV B/C 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. Different patterns of pre-S deletions were also found between the HBV/B and HBV/C groups according to different clinical outcomes. 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 ($P < 0.05$) [20]. Li, et al. [21] were found that the basic core promoter (BCP) and the preS/S regions of HBV isolated from 61 pairs of HBV/HIV co-infected and HBV mono-infected participants were analyzed. We found that the prevalence of HBV isolates with 17621T/1/764A and/or preS deletion mutations was 37.7%. 17621/1764A and preS deletions occurred more frequently in genotypes C and compared to genotype B ($p<0.05$). The basal core promoter/precore (BCP/PC) and complete surface (S) regions of HBV isolates were amplified and sequenced from 55 HCC cases and 22 non-HCC cancer controls. Phylogenetic analysis of 43 polymerase/complete S region amplicons showed that the majority (88.4%) clustered with subgenotype A1; 4.7% with A2; and 7% with A3. The 17621/1764A double mutation was detected in the majority (90.9%) of the isolates from HCC cases with preS deletions. Black HBV carriers were mainly infected with subgenotype A1; with HCC cases carrying BCP/PC and preS mutant strains that are associated with hepatocarcinogenesis [22]. The observed individual variance in development of HCC is attributable to differences in HBV genotype and mutations; host predisposing germline genetic variations; the acquisition of tumor-specific somatic mutations; as well as environmental factors. HBV genotype C and mutations in the preS; basic core promoter (BCP) or HBx regions are associated with an increased risk of HCC[23].Matsuo et al.[24] performed a detailed genetic analysis of 48 residents and four families from Binh Thuan Province; a southern coastal area of Vietnam. The HBV genotype was B4 in 91.7% and C1 in 8.3% of the cases. The intra-family s HBV sequence homology was high at 96.8-99.4%. The core region of all 20 isolates with genotype B4 was a recombinant of genotype C; and pre-S deletion was found in 20% of cases. The promoter mutation G1613A was found in 13.6% of cases; and a 24 bp insertion from nt1673 in the X region was found in 6.3% of cases. Moreover; the HBV genotype B4 isolates were found not only to be recombinants of genotype C; which results in a high cancer risk; but also to have other risk of HCC; pre-S deletions; the G1613A mutation; and X region insertions corresponding to the promoter. Pre-S mutations were identified by direct sequencing. Of the 265 subjects; 32 samples (12.1%) harbored pre-S deletion/insertion mutations. The prevalence of those pre-S mutations was 2.7% (2/75); 12.9% (8/62); 16.7% (11/66); and 17.7% (11/62) in asymptomatic carrier; chronic hepatitis; liver cirrhosis; and hepatocellular carcinoma groups; respectively. In HBV genotype B (HBV/B); pre-S1; pre-S1/S2; and pre-S2 deletion mutations were detected respectively in 3 (17.6%); 4 (23.5%); and 9 (52.9%) of 17 samples. On the other hand; in HBV/C; 12 of 15 samples (80.0%) showed a pre-S2 deletion mutation; and only 2 samples (13.3%) demonstrated a pre-S1/S2 deletion mutation. These results suggest that in HBV/B deletion mutation tends to occur in pre-S1 or pre-S1/S2 region; while in HBV/C the deletion mutation usually occurs in the pre-S2 region [25].

In this study; we found deletion mutation; insertion mutation or point mutation of HBV pre-S gene in hepatocellular carcinoma tissues or adjacent tissues; and whether this change is related to the molecular mechanism of HBV induced liver cancer? Further research is needed.

References

1. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, et al. (2015) Global cancer statistics, 2012. CA Cancer J Clin 65: 87-108.
2. Zhou M, Wang H, Zeng X, Yin P, Zhu J, et al. (2019) Mortality, morbidity, and risk factors in China and its provinces, 1990-2017: a systematic analysis for the Global Burden of Disease Study 2017. Lancet 394: 1145-1158.
3. Teng CF, Wu HC, Su IJ, Jeng LB (2020) Hepatitis B Virus Pre-S Mutants as Biomarkers and Targets for the Development and Recurrence of Hepatocellular Carcinoma. Viruses 12: 945.
4. Caligiuri P, Cerruti R, Icardi G, Bruzzone B (2016) Overview of hepatitis B virus mutations and their implications in the management of infection. World J Gastroenterol 22: 145-154.
5. Choi YM, Lee SY, Kim BJ (2019) Naturally occurring Hepatitis B virus mutations leading to endoplasmic reticulum stress and their contribution to the progression of hepatocellular carcinoma. Int J Mol Sci 20: 597.
6. Zhu P, Tan D, Peng Z, Liu F, Song L (2007) Polymorphism analyses of hepatitis B virus X gene in hepatocellular carcinoma patients from southern China. Acta Biochim Biophys Sin 39: 265-272.
7. Levrero M, Zucman-Rossi J (2016) Mechanisms of HBV-induced hepatocellular carcinoma. J Hepatol 64: S84-S101.
8. Yeung P, Wong DK, Lai CL, Fung J, Seto WK, et al. (2011) Association of hepatitis B virus pre-S deletions with the development of hepatocellular carcinoma in chronic hepatitis B. J Infect Dis 203: 646-654.
9. Chen BF (2018) Hepatitis B virus pre-S/S variants in liver diseases. World J Gastroenterol 24: 1507-1520.
10. Shen FC, Su IJ, Wu HC, Hsieh YH, Yao WJ, et al. (2009) A pre-S gene chip to detect pre-S deletions in hepatitis B virus large surface antigen as a predictive marker for hepatoma risk in chronic hepatitis B virus carriers. J Biomed Sci 16: 84.

11. Teng CF, Li TC, Huang HY, Lin JH, Chen WS, et al. (2020) Next-generation sequencing-based quantitative detection of hepatitis B virus Pre-S mutants in plasma predicts hepatocellular carcinoma recurrence. Viruses 12: 796-812.

12. Wang HC, Huang W, Lai MD, Su IJ (2006) Hepatitis B virus pre-S mutants, endoplasmic reticulum stress and hepatocarcinogenesis. Cancer Sci 97: 683-688.

13. Heo NY, Lee HC, Park YK, Park JW, Lim YS, et al. (2013) Lack of association between hepatitis B virus pre-S mutations and recurrence after surgical resection in hepatocellular carcinoma. J Med Virol 85: 589-596.

14. Kao JH, Liu CJ, Jow GM, Chen PJ, Chen DS, et al. (2012) Fine mapping of hepatitis B virus pre-S deletion and its association with hepatocellular carcinoma. Liver Int 32: 1373-1381.

15. Zhang AY, Lai CL, Huang FY, Seto WK, Fung J, et al. (2017) Deep sequencing analysis of quasispecies in the HBV pre-S region and its association with hepatocellular carcinoma. J Gastroenterol 52: 1064-1074.

16. Lin WL, Hung JH, Huang W (2020) Association of the Hepatitis B Virus Large Surface Protein with Viral Infectivity and Endoplasmic Reticulum Stress-mediated Liver Carcinogenesis. Cells 9: 2052.

17. Li X, Qin Y, Liu Y, Li F, Liao H, et al. (2016) PreS deletion profiles of hepatitis B virus (HBV) are associated with clinical presentations of chronic HBV infection. J Clin Virol 82: 27-32.

18. Lin YT, Jeng LB, Su IJ, Teng CF (2022) Approaches for Detection of Hepatitis B Virus Pre-S Gene Deletions and Pre-S Deleted Proteins and Their Application in Prediction of Higher Risk of Hepatocellular Carcinoma Development and Recurrence. Viruses 14: 428-437.

19. Zhang AY, Lai CL, Poon RT, Haung FY, Seto WK, et al. (2016) Hepatitis B virus full-length genomic mutations and quasispecies in hepatocellular carcinomal. J Gastroenterol Hepatol 31: 1638-1645.

20. Chen BF (2016) Different pre-S deletion patterns and their association with hepatitis B virus genotypes. World J Gastroenterol 22: 8041-8049.

21. Li KW, Kramvis A, Liang S, He X, Chen QY, et al. (2017) Higher prevalence of cancer related mutations 1762T/1764A and PreS deletions in hepatitis B virus (HBV) isolated from HBV/HIV co-infected compared to HBV-mono-infected Chinese adults. Virus Res 227: 88-95.

22. Mak D, Kramvis A (2020) Molecular characterization of hepatitis B virus isolated from Black South African cancer patients, with and without hepatocellular carcinoma. Arch Virol 165: 1815-1825.

23. An P, Xu J, Yu Y, Winkler CA (2018) Host and viral genetic variation in HBV-related hepatocellular carcinoma. Front Genet 9: 261.

24. Matsuo J, Do SH, Yamamoto C, Nagashima S, Chuon C, et al. (2017) Clustering infection of hepatitis B virus genotype B4 among residents in Vietnam, and its genomic characters both intra- and extra-family. PLoS One 12: e0177248.

25. Utama A, Siburian MD, Fanany I, Intan MD, Dhenni R, et al. (2011) Low prevalence of hepatitis B virus pre-S deletion mutation in Indonesia. J Med Virol 83: 1717-1726.