Biomedical Sciences
2021; 7(4): 120-123
http://www.sciencepublishinggroup.com/j/bs
doi: 10.11648/j.bs.20210704.15
ISSN: 2575-3924 (Print); ISSN: 2575-3932 (Online)

Naturally Occurring Genetic Deletions or Insertions in Basal Core Promoter and PreS1 Region Do Not Critically Affect Hepatitis B Virus Replication

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To cite this article:

Yihan Xiao, Zongxin Li, Xiuji Cui, Yunfan Quan. Naturally Occurring Genetic Deletions or Insertions in Basal Core Promoter and PreS1 Region Do Not Critically Affect Hepatitis B Virus Replication. *Biomedical Sciences*. Vol. 7, No. 4, 2021, pp. 120-123.
doi: 10.11648/j.bs.20210704.15

Received: December 11, 2021; **Accepted**: December 23, 2021; **Published**: December 29, 2021

Abstract: Objective: Naturally occurring genetic deletions and insertions in basal core promoter (BCP) and PreS1 region of hepatitis B virus (HBV) were frequently detected in sera of chronic hepatitis B patient and these genetic variants are related to the poor response to antiviral treatment as well as the progression of liver cirrhosis and hepatocellular carcinoma. Thus, the aim of the study was to understand the effect of genetic deletions and insertions in BCP and PreS1 region on viral replication competence. Methods: HBV DNA was extracted from the sera of a chronically HBV infected patient and the whole HBV genome was amplified by PCR method. The genome was then cloned into the pCDNA3.1 vector to construct HBV-expressing plasmid, which were used to transfect HepG2 cells. The intracellular HBV DNA was detected by Southern blot method. Results: All cloned HBV genomes carried genetic deletion or insertion in the BCP and PreS1 region. However, these mutations did not seem to affect the competence of viral replication. Conclusion: Although the genetic deletion reported here may not affect the HBV replication, patients with such genetic variation should be carefully monitored during the therapy because deletions in the BCP and PreS1 are associated with progression of liver disease.

Keywords: Basal Core Promoter, Hepatitis B Virus, PreS1, Viral Replication

1. Introduction

Hepatitis B virus (HBV) mainly infects hepatocytes, causing hepatitis, which may lead to liver cirrhosis (LC) and hepatocellular carcinoma (HCC) [1]. It has been estimated that approximately 7.18% of people in China are positive to hepatitis B surface antigen (HBsAg) [2-4]. Although current anti-HBV drugs efficiently inhibit the viral replication, they cannot cure the HBV infection [5, 6].

HBV belongs to *Hepadnaviridae* and its genome is partially relaxed circular (rc) double stranded DNA [7]. Upon the infection, the rcDNA is transported to nucleus of hepatocyte to form covalently closed circular (ccc) DNA, which is the template for all HBV RNA transcription [8-10]. Among HBV RNAs, pregenomic (pg) RNA is the template for HBV DNA synthesis. It had been reported that the transcription pgRNA was regulated by viral core promoter located between nucleotide (nt) 1575 and nt 1849 [11]. The core promoter contains an upper regulatory region (nt 1613-1742) and a basal core promoter (BCP) (nt 1743-1849) [12, 13]. Recent

studies showed that genetic mutation in BCP region, such as A1762T and G1764A mutations, enhance HBV replication ability and reduce the production of HBeAg [14-17]. Moreover, mutations in BCP are related to the progression of LC and HCC [14, 16, 18]. PreS1, coupled with preS2 and Surface gene, encodes the large surface protein of HBV [8]. It has been reported that the surface protein regulates the HBV replication [19-23].

In this study, the genetic deletions or insertions in the BCP and PreS1 region were frequently found in the sera of chronic hepatitis B patient who had not yet received anti-HBV treatment. Therefore, we study the effect of genetic deletions or insertions in these regions on HBV replication.

2. Materials and Methods

2.1. Extraction of Serum HBV DNA

HBV DNA was isolated from 200 ul of a serum of chronically HBV- infected patient using QIAamp MinElute Virus Spin Kit (Qiagen, Germany, Cat#5704) according to the manufacture's instruction. The present study was approved by the Ethics Committee of the Hainan Medical University, Haikou, Hainan Province, China.

2.2. Construction of HBV Expressing Plasmid

To construct the 1.05 copies of HBV DNA that could produce viral particles, the whole HBV genome and a short fragment of HBV genome from nt 1799 to 1988 was amplified by F-1818/L-reverse primer and S-forward (*Sap*I) and S-reverse (*Hind*III), respectively, by KOD DNA polymerase (TOYOBO, Japan, Cat#KMM101), which has proofreading activity to minimize nucleotide mismatch (Table 1). Then, the PCR products were extracted from the 1% agarose gel and were subsequently double-digested with restriction enzyme *Sac*I and *Sap*I to release the HBV DNA. The short fragment was double digested with *Sap*I and *Hind*III. Released DNAs were recovered again from 1% agarose gel. The full-length and short fragment of digested HBV DNA were co-ligated with pCDNA 3.1 (+) digested with *Sac*I and *Hind*III and then, transform into DH5α *Escherichia coli* (*E.Coli*). Seven colonies were further cultured in Luria-Bertani (LB) medium with ampicillin (100ug/ml), then plasmids (denoted pCDNA3.1-HBV C1, C2, C3…, C7) were isolated and analyzed with DNA sequencing. The alignment of sequences was performed by the Clustal W method using Molecular Evolutionary Genetics Analysis (MEGA) software, version X.

Table 1. *List of PCR primer pairs.*

	Primers	Sequence (5'→3')	Nucleotide position
HBV Plasmid			
Full length	F-1818 (*Sac*I)	5'-CTATATTAAGCAGAGCTCCACCAGCACCATGCAACTTTTTC-3'	nt 1804-1826
	L-Reverse (*Sap*I)	5'-CCGGAAAGCTTGAGCTCTTCAACACACCAATTTATGCCTAC-3	nt 1783-1803
Short fragment	S-Forward (*Sap*I)	5'-CCGGAAAGCTTGAGCTCTTCGTGTTCACCAGCACCATGCA-3	nt 1799-1818
	S-Reverse (X*ho*I)	5'-CCGGAAAGCTTGAGCTCTTCAACACACCAATTTATGCCTAC-3	nt 1967-1988
For sequencing	pCDNA3.1 forward	5'-CTAGAGAACCCACTGCTTAC-3'	cover nt1804
	P4	5'-CCTTGGACACATAAGGTGGG-3'	nt 2457-2476
	P5	5'-GTGGAGCCCTCAGGCTCAGG-3'	nt 3075-3094
	P6	5'-GCTGCTATGCCTCATCTTC-3'	nt 415-433
	P7	5'-CCAACTTACAAGGCCTTTC-3'	nt 1102-1120

2.3. Cell Culture and Transient Transfection

Human hepatoblastoma (HepG2) cells were maintained with a high-glucose DMEM supplement with 10% (v/v) of fetal bovine serum (FBS) (Biological industries, Cat# BISH2177) and 50 ug/ml of penicillin-streptomycin at 37°C and 5% CO_2. HepG2 cells seeded to 60-mm culture dishes were transfected with 4 μg of plasmid DNAs using lipofectamine 3000 (Thermofisher, Cat# L3000-008). Cells were collected on day 4th post-transfection for determination of HBV replicating competency.

2.4. Detection of HBV Replicative Intermediates

HBV replicative intermediates inside the cytoplasmic nucleocapsid were released by protease and sodium dodecyl sulfate (SDS) treatment and were detected by southern blot, as previously described [24]. Briefly, HepG2 cells were lysed by 200 μl of NP-40 lysis buffer and centrifuged at 12,000xg for 5 min to remove the nuclear pellet. The cytoplasmic lysate (20 ul) was incubated in 30 μl of reaction containing 15 mM of ethylene diamine tetraacetic acid (EDTA), 0.5% SDS and 0.5 ug of proteinase K (PK) at 37°C for 2 hours. The digested lysate was then resolved on 1% agarose gel electrophoresis and transferred onto the nylon membrane. HBV replicative intermediates on the membrane were probed by digoxigenin (DIG)-labeled HBV DNA probe using DIG High Prime DNA labeling and Detection Starter Kit II (Roche, Cat#11585614910). Intracellular HBV DNA was isolated from HepAD38 cells, an HBV expressing cell, and used as positive contraol.

3. Results

3.1. Detection of Genetic Deletion in BCP and PreS1 Region

In this study, we detected the deletion of nt1759-1767 deletion in BCP region in all seven colonies (Figure 1A). Clone C3 also had a 14-nt insertion between nt1638 and 1639 and C5 had a 13-nt insertion between nt1620 and 1621. In the PreS1 region, all colonies defective start codon and, except C1 and C2, all had deletion between nt3071 to nt3199, which encode amino acid (aa) from 76 to 118 of PreS1 (Figure 1B).

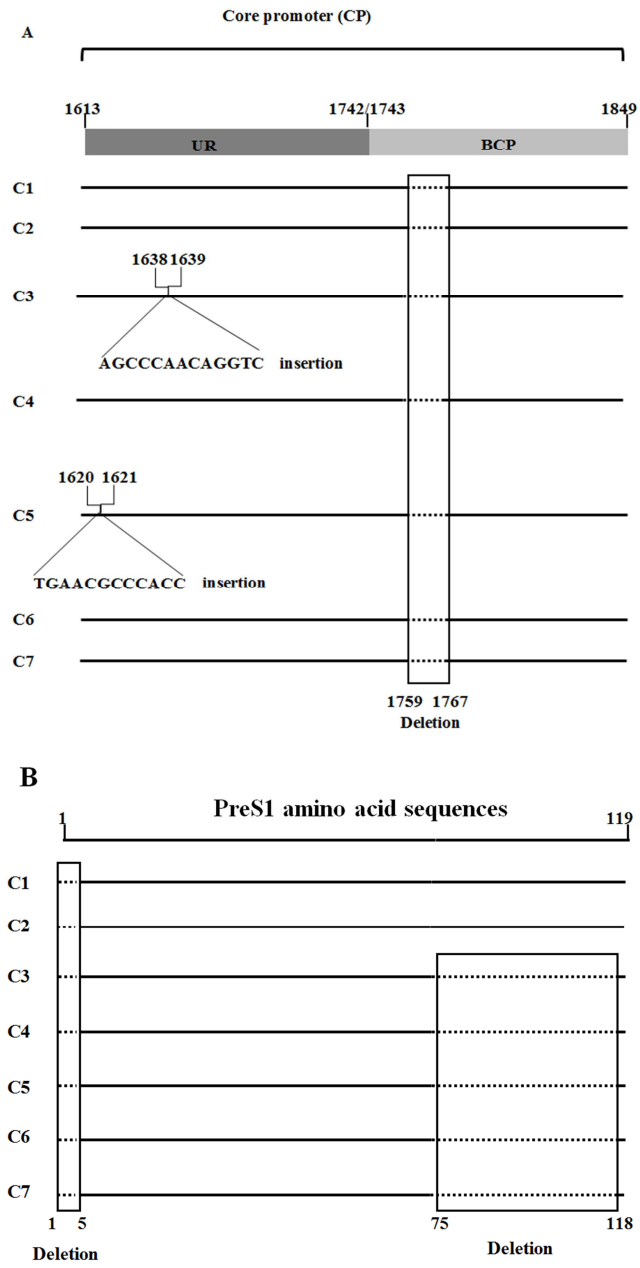

Figure 1. *Schematic diagram of genetic variation in BCP and PreS1. The full-length sequence of HBV was aligned with Clustal W method. A, the genetic variation of BCP. B, amino acid deletion in PreS1 region.*

3.2. Genetic Deletion or Insertion in BCP and PreS1 had Minor Effect on HBV Replication

To study the effect of genetic variation found in the BCP and PreS1 region on HBV replication, HBV-expressing plasmid was constructed and used to transiently transfect HepG2 cells. The intracellular HBV DNA was isolated and examined with Southern blot. The results showed that the replication of HBV DNA was varied in different colonies, but did not seem to be greatly affected by the genetic variations. Among the seven colonies analyzed, C4 and C7 showed the strong replicative capability, whereas that of C2 and C3 was weak.

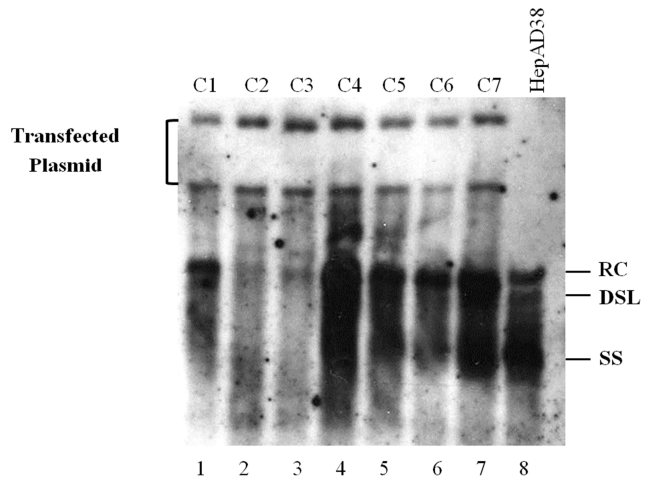

Figure 2. *Intracellular HBV DNA synthesis. Intracellular HBV DNA was extracted and detected by Southern blot. Abbreviations: RC, relaxed circular DNA; DSL, double stranded linear; SS, single stranded.*

4. Discussion

HBV is an DNA virus and the pgRNA is known to the template for HBV DNA synthesis [8]. Recently, several studies have shown that the BCP regulates the expression of pgRNA, and the genetic variation in BCP can enhance the viral replication [13, 14]. Here, we detected varying levels of HBV replication in mutants with genetic deletions or insertions in BCP, which suggested that the genetic variation do not critically affect viral replication. However, because the region nt1757-1767 overlaps HBV X gene (nt 1374-1835), a deletion here might affect the structure and function of the X protein [25, 26]. The X protein is a protein with multifunction and is associated with the development of LC and HCC [26].

Previous studies have shown that the deletions in the PreS1 region may cause the intracellular accumulation of HBV DNA and increase the expression of X protein [20-23, 26]. In the present study, overall viral replication levels of all colonies with a PreS1 deletion (C4, C5, C6 and C7), except C3, were higher than C1 and C2. However, considering the low replication level of HBV in C3 and the varying replication levels of C1 and C2, deletion in PreS1 region might have only a minor effect on the viral replication.

5. Conclusion

In the present study, we detected genetic variations in the BCP and PreS1 region of the HBV genome, but they had only minor effects on its replication. However, because the BCP and X gene sequences overlap, patients infected with these genetic variants should be monitored carefully.

Conflict of Interest

All authors declare that they don't have any conflict of interest.

Acknowledgements

This work was supported by Hainan Provincial Natural Science Foundation of China (Grant No. 819QN237 for FQ, 820RC640 for XC), the National Natural Science Foundation of China (Grant No. 81660335, 82060365) for XC, and Startup Foundation of Hainan Medical University for XC.

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