

Hepatitis B Virus A1762T/G1764A Mutation Has a Minor Effect on the Viral Spliced DNA Expression

Zongxin Li^{1,†}, Yihan Xiao^{2,†}, Yuanhai Zhou¹, Ting Tang¹, Pin Yang¹, Long Sun^{5,*}, Xiuji Cui^{1,3,4,*}

¹Department of Pathogenic Biology, School of Basic Medicine and Life Science, Hainan Medical University, Haikou, China

²Department of Clinical Laboratory, The Second Affiliated Hospital of Hainan Medical University, Haikou, China

³Key Laboratory of Tropical Translational Medicine of Ministry of Education, Hainan Medical University, Haikou, China

⁴Hainan Medical University-The University of Hong Kong Joint Laboratory of Tropical Infectious Diseases, Hainan Medical University, Haikou, China

⁵Department of Infectious Disease, The First Affiliated Hospital of Hainan Medical University, Haikou, China

Email address:

cuij26@hainmc.edu.cn (Xiuji Cui), hy0203068@hainmc.edu.cn (Long Sun)

*Corresponding author

† Zongxin Li and Yihan Xiao are co-first authors.

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Abstract: Background: Hepatitis B virus (HBV) is the major cause of chronic hepatitis B, which can lead to liver cirrhosis and hepatocellular carcinoma. During HBV replication, splicing of viral RNA frequently occurs, and the spliced RNA or DNA has been reported to be related to the development of liver disease. HBV transcription is mainly regulated by core promoter ranging from nucleotide 1613 to 1849 and the A1762T/G1764A mutation, which can increase the viral transcription, is frequently detected in this region. Objective: The study aimed to analyze the effect of the A1762T/G1764A mutation in the core promoter of HBV on viral RNA splicing. Methods: The wild type (WT) and the A1762T/G1764A mutant HBV genome were cloned into the pCDNA3.1 vector, which was then transiently transfected into HepG2 cells. The intracellular HBV DNA and core protein were determined by the Southern blot and the Western blot, respectively. The relative level of HBSD was examined by quantitative PCR using TB green reagent. Results: the A1762T/G1764A mutation could enhance the ability of viral replication, however, had a minor effect on HBSD expression. Conclusion: The double mutation in CP could regulate the viral transcription, but was not involved in the viral RNA splicing.

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

1. Introduction

Over 300 million people are suffering from Hepatitis B virus (HBV) infection, which mainly establishes infection in hepatocytes, and further causes hepatitis, liver cirrhosis (LC), and hepatocellular carcinoma (HCC) worldwide [1]. China is one of the HBV-endemic countries with approximately 100 million hepatitis B surface antigen (HBsAg) positive people [2-4]. So far, there are several anti-HBV drugs available to efficiently block viral replication, however, none of them can eliminate HBV from the human body [5, 6].

HBV is a member of *Hepadnaviridae*. Upon the infection, the HBV genome, a partially double-stranded relaxed circular

(rc) DNA, is delivered to the nucleus to form covalently closed circular (ccc) DNA, which serves as the template for all HBV RNA transcription [7-10]. An HBV transcript called pregenomic (pg) RNA serves as the template for HBV DNA synthesis. The transcription pgRNA is regulated by the viral core promoter (CP) ranging between nucleotide (nt) 1575 and nt1849 [11]. Studies have shown that genetic mutation in CP region, especially Adenine (A) to Thymine (T) mutation at nt1762 (A1762T) and Guanidine (G) to Adenine (A) mutation at nt1764 (G1764A) could enhance the ability of HBV replication and decrease the HBeAg production [12-15].

Moreover, the double mutation is related to the development of LC and HCC [12, 14-16].

Like eukaryotes, several viruses also undergo mRNA splicing to increase viral RNA diversity. Alternative splicing also happens during HBV pgRNA transcription (normally deleted between nt 2447-1-489, Figure 1) and is regulated by CP region. The spliced pgRNA also can synthesize the HBV

spliced DNA, denoted HBSD. So far, little is known about the regulation of the CP region, especially the A1762T/G1764A double mutation in viral RNA splicing.

Thus, here, we study the effect of the double mutation in the CP region on the expression of spliced pgRNA by quantifying the intracellular HBSD expression.



Deletion of the HBV genome commonly located at the region ranging from nt2447 to nt489.

Figure 1. Illustration of a common region of genetic deletion in HBV genome.

2. Materials and Methods

2.1. Construction of HBV Expressing Plasmid

To construct the HBV-producing plasmid DNA, HBV DNA was first isolated from the serum (200 µl) of a chronic hepatitis B patient using QIAamp MinElute Virus Spin Kit (Qiagen, Germany, Cat#5704). The procedure of sampling was approved by the Ethics Committee of the Hainan Medical University, Haikou, Hainan Province, China.

Then, the full-length (nt1804-nt1803) and a short fragment (nt1799-nt1988) of HBV DNA were amplified using F-1818 (5'-CTATATTAAGCAGAGCTCCACCAGCACCATGCAACCTTTTC-3')/L-reverse (5'-CCGGAAAGCTTGAGCTCTTCAACACACCAATTATGTCCTAC-3') primer and S-forward (*SapI*) (5'-CCGGAAAGCTTGAGCTCTTCGTGTTCCACCAGCACATGCA-3') and S-reverse (*HindIII*) (5'-GATTCCCTCGAGAAGCTTGATCTCGAACAGAAGGAAAGAA-3'), respectively, using KOD DNA polymerase (TOYOBO, Japan, Cat#KMM101) possessing proof-reading activity.

PCR products recovered from agarose gel were double-digested with restriction enzymes *SacI* and *SapI* to release the full-length HBV DNA. The short fragment was released by *SapI* and *HindIII* double digestion. Released DNAs were isolated from 1% agarose gel. Subsequently, the digested full-length and short fragment HBV DNA was ligated with *SacI* and *HindIII* digested pCDNA 3.1(+) and then, transform into DH5α *Escherichia coli* (*E. Coli*) by standard cloning method. One of colonies was further cultured in Luria-Bertani (LB) medium containing ampicillin (100ug/ml), then plasmids (denoted pCDNA3.1-HBV WT) were isolated and analyzed with DNA sequencing.

To generate the A1762T/G1764A double mutation, PCR based site-directed mutagenesis method was employed. In brief, 50 ng of pCDNA3.1-HBV WT was amplified with

BCP-F

(5'-GGAGATTAGGTTAATGATCTTTGTACTAGGA-3', nt1748-1778) and BCP-R (5'-TCCTAGTACAAAGATCATTAACCTAATCTCC-3', nt1748-1778) by 12 cycles using KOD DNA polymerase. The PCR product was directly digested with *DpnI* enzyme to remove the plasmid DNA, and then, was subjected to the standard conventional cloning. The A1762T/G1764A double mutation was confirmed by Sanger sequence analysis and the plasmid DNA was denoted pCDNA3.1-HBV A1762T/G1764A.

2.2. Cell Culture and Transient Transfection

Human hepatoblastoma (HepG2) cells were cultured in a high-glucose DMEM 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% CO₂. Four micrograms of plasmid DNA were transiently transfected into HepG2 cells (60-mm culture dish) with X-TremeGENE HP DNA transfection reagent (Roche, Cat# 06366236001). On day 4th post-transfection, cells were harvested by NP-40 lysis buffer for further analysis.

2.3. Isolation and Detection of Intracellular HBV DNA

Intracellular HBV DNA inside the cytoplasmic nucleocapsid was released by protease and sodium dodecyl sulfate (SDS) treatment and was further isolated by the phenol/chloroform extraction method and the viral DNA was detected by the standard Southern blot method, as previously described [17]. Briefly, HepG2 cells were lysed by 500 µl of NP-40 lysis buffer and centrifuged at 12,000xg for 5 min to remove the nuclear pellet and saved 100 µl of lysate for detection of HBV core protein expression. The cytoplasmic lysate (400 µl) was treated with 20 units of micrococcal nuclease for 1hr to remove nucleic acid outside the nucleocapsid. Then, the nuclease was inactivated by 15 mM of ethylene diamine tetraacetic acid (EDTA). Subsequently, the lysate was incubated with 0.5% SDS and 0.5 ug of proteinase

K (PK) at 37°C for 2 hours to release HBV DNA from the nucleocapsid. The digested lysate was then subjected to standard phenol/chloroform extraction to purify the HBV DNA. Purified intracellular DNA was resolved on 1%-agarose gel electrophoresis and transferred onto the nylon membrane. The intracellular HBV DNA on the membrane was probed by a digoxigenin (DIG)-labeled HBV DNA probe using DIG High Prime DNA labeling and Detection Starter Kit II (Roche, Cat#11585614910).

2.4. Detection of HBV Core Protein

Twenty microliters of NP-40 lysed cells were denatured at 95°C for 10min and chilled on ice for 5 min. Then, the lysate was separated by 15% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by Western blotting. Antibodies against HBV core protein (Cat# 1AHC24) were purchased from Institute Immunology Co. LTD (Japan) and β -ACTIN (Cat#sc-47778) was purchased from Santa Cruz Biotech. The level of β -ACTIN expression was used to normalize cell numbers in each experiment group. The chemiluminescent signal of core protein and β -ACTIN on blot was detected with the Tannon ChemDoc system and the density of the signal was measured by ImageJ software (<https://imagej.nih.gov/ij/index.html>).

2.5. Quantitative Real-Time PCR

The amount of intracellular full-length HBV DNA and HBSD from each group was quantified by the Quantitative polymerase chain reaction (qPCR) method using TB green premix Ex Taq II (Takara, Cat#RR820A). The full-length HBV DNA was amplified with primer WT-F

(5'-TCTAGACTCGTGGTGGACTTCTCTC -3', nt 249-273) and WT-R (5'-CATAGCAGCAGGATGAAGAGGAA-3', nt403-425); the HBSD was amplified by SP-F (5'-CCGCGTCGCAGAAGATCT-3', nt2413-2430) and SP-R (5'-ATGGGAATACAGGTGCAGTTTCC-3', nt589-611), as previously described [18]. The data was validated by primer specificity in a melting curve analysis.

2.6. Statistic Analysis

Experiments were repeated six times and the differences in means between the groups were analyzed by independent *t*-test using Prism Graph software. A *P* value of <0.5 was considered statistically significant.

3. Results

3.1. Effect of A1762T/G1764A Mutations on the HBV Replication

To study the effect of A1762T/G1764A double mutations on the HBV replication, pCDNA3.1-HBV WT, and pCDNA3.1-HBV A1762T/G1764A were transiently transfected into the HepG2 cells. The level of HBV replication was evaluated by the intracellular HBV DNA and core protein expression.

As a result, the level of both HBV intracellular DNA and core protein were dramatically increased in cells transfected with pCDNA3.1-HBV A1762T/G1764A compared with pCDNA3.1-HBV WT and differences in expression level were statistically significant (Figure 2).

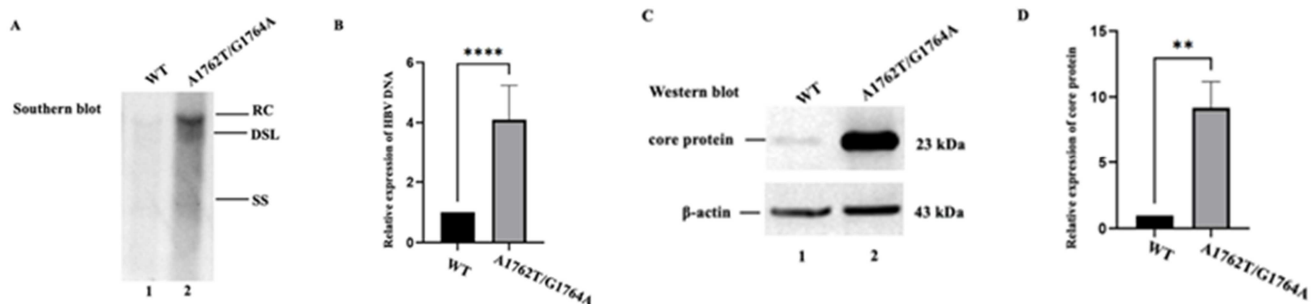


Figure 2. Expression of intracellular HBV DNA and core protein.

HBV-expression plasmid constructs were transfected to HepG2 cells and the intracellular DNA was detected by the Southern blot and the core protein was detected by the Western blot. A, intracellular DNA detected by the Southern blot; B, relative expression of total intracellular HBV DNA between groups. C, intracellular core protein detected by the Western blot; D, relative expression of intracellular core protein expression between groups. Abbreviations: WT, wild type; RC, relaxed circular; DSL, double-stranded linear; ss, single-stranded.

3.2. Effect of A1762T/G1764A Mutations on HBSD Expression

To study the effect of A1762T/G1764A mutations on HBSD expression, HBV expressing plasmid with either wild type and A1762T/G1764A mutation was transiently transfected to HepG2 cells. The level of intracellular full-length HBV DNA and HBSD was determined by qPCR.

To compare the difference in HBSD expression between groups, the level of HBSD was shown as the ratio of the amount of HBSD to full-length HBV DNA in each group. The level of full-length HBV DNA was used as the internal control.

As a result, the A1762T/G1764A double mutation seemed to slightly decrease the expression of HBSD, however, there was no statistically significant (Figure 3).

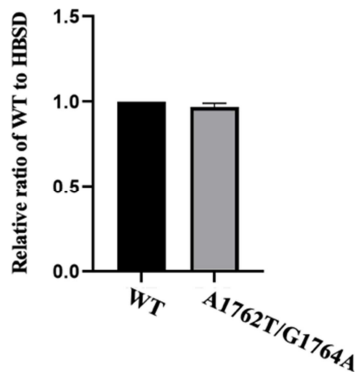


Figure 3. Determination of HBSD expression by qPCR.

HBV-expression plasmid constructs were transfected to HepG2 cells and the intracellular DNA was isolated after treatment of proteinase K and SDS, followed by phenol/chloroform extraction. The full-length and HBSD in isolated intracellular HBV DNA were determined by qPCR. The level of HBSD was normalized to the level of full-length DNA in the same group. Abbreviations: WT, wild type

4. Discussion

HBV DNA replicates via reverse transcription and the pgRNA is known to be the template for HBV DNA replication [8]. Recent studies have shown that the expression of pgRNA is regulated by the CP region. The CP contains an upper regulatory region (nt 1613-1742) and a basal core promoter (BCP) (nt 1743-1849) and the genetic variation in BCP, such as A1762T/G1764A, can up-regulate viral replication [12, 19, 20]. In addition, the part of BCP (nt1757-1767) overlaps HBV X gene (nt 1374-1835), which codes a multifunctional protein regulating viral replication [21]. Thus, the A1762T/G1764A may also affect the structure and function of the X protein [23].

RNA splicing is a common event in microbes, such as the human immunodeficiency virus (HIV), human papillomavirus (HPV), and HBV. Unlike HIV and HPV, RNA splicing is not required for HBV replication [22]. However, the HBV spliced RNA or its product, HBSD, may modulate viral replication and develop LC and HCC. So far, there is limited information about the association between CP and HBSD.

Here, we attempted to study the regulation of HBSD expression by CP through the A1762T/G1764A double mutation. Consistent with previous studies, the double mutation dramatically increased viral replication, suggesting that the CP region is regulating viral transcription. However, the A1762T/G1764A double mutation seemed not to affect the HBSD expression in the present study, suggesting the CP may not involve in the RNA splicing during the HBV life cycle.

5. Conclusion

HBV pgRNA splicing is a common process during HBV life cycle, and some spliced pgRNA can further synthesize spliced DNA that may be associated with the development of end-stage liver disease. The transcription of pgRNA is

regulated by the HBV core promoter. The A1762T/G1764A double mutation, which can increase the viral replication, is the most common mutation in core promoter, and the double mutation was assumed to be related to the pgRNA splicing. However, in our study, the double mutation seemed to have a minor effect on the expression of spliced pgRNA, suggesting there is no much correlation between the core promoter mutation and HBV RNA splicing.

Conflict of Interest

All authors declare that they have no conflict of interest.

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