REPLICATION EFFICIENCY AND SEQUENCE ANALYSIS OF FULL-LENGTH HEPATITIS B VIRUS ISOLATES FROM HEPATOCELLULAR CARCINOMA TISSUES

Xu LIN, Zhang-Mei MA, Xin YAO, Yan-Ping ZHANG and Yu-Mei WEN*
Department of Molecular Virology, Medical Center of Fudan University, Shanghai, China

Prolonged replication of hepatitis B virus (HBV) in liver tissues of hepatitis B patients has been considered as an important risk factor for the development of malignancy. Few studies on full-length HBV sequencing in association with the replication efficiency of isolates from HCC tissues have been reported. To study the structural and functional genomics of HBV isolates from Chinese hepatocellular carcinoma (HCC) patients, full-length HBV genomes were amplified from 6 HBV-marker positive HCC tissues and used to transfect HepG2 cells. Five of 6 isolates showed high replicative efficiency. All isolates were of genotype C and the mutations in the B cell and T helper (Th) cell epitopes of the envelope and the core region. In addition, the X region of 2 isolates contained a stop codon mutation that was predicted to result in a truncated X protein. High replicative HBV immune escape mutants could be important factors to initiate pathological processes for the development of HCC in Chinese patients.

Key words: hepatitis B virus; hepatocellular carcinoma; replication

Hepatitis B virus (HBV) belongs to the Hepadnaviridae family, which is unique in possessing a partially double-stranded DNA genome. This genome consists of 4 open reading frames encoding the envelope antigen (S/Pre-S), the core antigen (C/Pre-C), the viral polymerase (P) and a multifunctional transcriptional transactivator, the X protein. In HBV endemic areas, chronic HBV infection is closely associated with hepatocellular carcinoma as more than 90% of Chinese hepatocellular carcinoma (HCC) patients have been found positive for HBV-markers. Although it is generally accepted that carcinogenesis is a multi-step event, the functional properties of HBV strains involved in the early stage of carcinogenesis have not been fully explored. In a previous study, we compared the nucleotide sequence and replicative efficiency of sequential HBV isolates from a Chinese patient who progressed from HBV asymptomatic carrier to hepatocellular carcinoma and showed that post-HCC HBV isolates possessed enhanced replicative efficiency. High replicative HBV strains might be associated with HCC if they led to increased number of infected hepatocytes, resulting in severe liver injury mediated by host immune response; to emergence of mutants that would escape from host immune surveillance; or to increased rate of integration of viral DNA. To further investigate the incidence and possible roles of high replicative immune escape mutants derived from HCC tissues, we describe the structural and functional analysis of full-length HBV genomes from 6 independent Chinese HCC tissues.

MATERIAL AND METHODS

Liver cancer tissue samples
Liver cancer tissues from serum HBsAg-positive hepatocellular carcinoma patients were collected after resection of the tumor and the diagnoses were confirmed by histopathological examination. Sample collection was in accordance with Chinese State Ethnic Regulations. These tissues were snap-frozen in liquid nitrogen until the extraction of DNA.

Extraction of DNA
As reported previously, 100 mg of frozen liver cancer tissues were washed with PBS for 5 times and homogenized in 1.5 ml of PBS. After centrifugation at 5,000 rpm for 10 min, pellets were resuspended in 1.2 ml of lysing buffer (50 mM Tris-HCl pH 8.0, 100 mM EDTA, 1% SDS, 400 μg/ml protease K, 100 μg/ml RNaseA) incubated at 55°C for 16 hr. DNA was extracted with phenol/chloroform (1:1) once, subsequently with chloroform once, precipitated with ethanol and dissolved in 200 μl of sterile distilled water.

Amplification and cloning of full-length HBV DNA
Extracted DNA was amplified using “hot-start” PCR using high fidelity Taq DNA polymerase (Boehringer-Mannheim, Mannheim, Germany) to yield full-length HBV DNA, as described by Gunther et al. The nucleotide sequences of the primers used were as follows: sense primer, 5' - GCGGAAAGCTTGGCTCTCTCACCCTCTGGTCTCA-3'; antisense primer, 5' - GGGAAAGCTTGGCTCTCTCACCCTCTGGTCTCA-3'; 3' PCR was carried out at 94°C for 30 sec, 60°C for 1 min, 68°C for 3 min for the first 10 cycles and from 11–35 cycles, the extension time at 68°C was increased by 5 sec at each cycle. After 35 cycles, the final extension time was 7 min at 72°C. PCR products were separated on 0.7% agarose gel and 3.2 Kb fragments were recovered and digested with SstI for 6 hr, ligated to vector pUC18 and used to transfect DH5α cells. Positive clones were selected and checked by digestion with SstI and HindIII enzymes.

Sequence analysis of full-length HBV genome
Sequencing was done by automated sequencer (ABI 377) using pUC 18 universal primers, sense and antisense primers as previously reported. Using Vector NTI 5.5, Bioedit and Tree-View 1.5 software (Table I). Genotyping of the isolates was carried out using the standard A–F genotype HBV genomes as references (genotype A, X02763; genotype B, D00329; genotype C, M12906; genotype D, X02496; genotype E, X75664; genotype F, X75663). Prediction of the sero-types of the HBV isolates was based on the amino acids at positions 122 and 160 of the S antigen. Structural analysis of the 6 isolates in our study was based on comparison with the 10 full-length genomes of HBV genotype C serotype adr deposited in GenBank (m12906, AB042285, AB042284, AB042283, AB042282, AB026815, AB026814, AB026813, AB026812, AB026811).

Transfection of Hepg2 cells with full-length HBV clones
Recombinant DNA from each isolate was extracted and purified with Qiagen maxiprep kits (Qiagen, Germany) and HBV DNA was

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*Correspondence to: Medical Center of Fudan University, Department of Molecular Virology, 138 Yi Xue Yuan Road, Shanghai, 200032, China. Fax: +86086-21-6474578. E-mail: ymwen@shmu.edu.cn

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excised from the recombinant plasmids by digestion with SapI (New England Biolabs, Beverly, MA) at 1 U/μg of DNA. DNA from each isolate was used to transfect HepG2 cells as reported previously.2 In brief, 10 μg of HBV DNA were transfected into HepG2 cells in 60 mm plates by calcium phosphate precipitation method. Duplicate plates were used for all samples and 5 μg of reporter plasmid DNA expressing secreted alkaline phosphatase (SEAP) were cotransfected as an internal control for the normalization of transfection efficiency between plates. Culture supernatants and cells were collected separately at 72 hr after transfection. Vector pUC 18 DNA was used as a mock transfection control.

Purification and collection of HBV DNA from intracellular core particles

As reported previously,2 intracellular core particles were collected, purified by centrifugation and precipitated with polyethylene glycol, digested with DNase I to ensure digestion of any remaining DNA used for transfection. Core particles were digested with proteinase K to release HBV DNA, followed by extraction and precipitation in ethanol.

Southern blotting and HBV DNA hybridization

Before electrophoresis, the quantity of intracellular core HBV DNA used to load gels from each sample was adjusted according to the transfection efficiency. For the normalization of transfection efficiency, SEAP expression levels from all culture supernatants were assayed by alkaline phosphatase activity versus its substrate. In brief, 100 μl of culture supernatant from each plate of transfected cells were collected, heated at 65°C for 5 min to inactivate endogenous phosphatase, followed by incubating the supernatant with the substrate (120 mM paranitrophenol in SEAP buffer) for 15 min. The level of SEAP was determined by ELISA at 405 nm (λmax). The plate that

![FIGURE 1](image)
expressed the lowest level of SEAP was arbitrarily set as 1 and the levels of SEAP expressed in other plates were compared to this plate at a relative ratio. According to the ratio of SEAP from each sample, the quantity of intracellular core HBV DNA used for electrophoresis was adjusted (the ratio of transfection efficiency between plates varied from 1.00–1.10). After electrophoresis, intracellular core HBV DNA was blotted onto Hybond N\(^{-}/\)H11001 nylon membrane (Amersham, Buckinghamshire, UK) as described.\(^5\) Intensity of hybridization signal was scanned by densitometry (Image analysis version 3.5 software, Kodak EDAS 290 system).

**HBsAg and HBeAg assays**

HBsAg and HBeAg in the supernatant of cell cultures were assayed by Abbott EIA kits (North Chicago, IL).

**RESULTS**

**Genotyping**

The 6 full-length HBV genomes amplified from HCC tissues were compared to the known HBV full-length sequences of A–F genotypes. All 6 isolates, namely: #14, #15, #16, #20, #97 and #AID (numbered previously as sD in Reference 2) belong to genotype C (GenBank accession numbers: AF411408, AF411409, AF411410, AF411411, AF411412 and AF 182804 respectively).

**Mutations in HBV coding regions**

Altogether there were 7 identified “hot-spots” of amino acid mutations/deletions in the envelope protein (pre-S1, pre-S2 and S). As shown in Figure 1, 67% (4/6) of the HBV isolates had mutations at the 10th codon of pre-S1 (Q\(\rightarrow\)K/H). In pre-S2, 83% (5/6) showed mutation/deletion between codons 131–142. These “hot-spots” were located in the reported B cell epitopes of pre-S1 and pre-S2 (pre-S1 B cell epitope was reported at codons 1–26, pre-S2 B cell epitope was reported at codons 134–144).\(^6,7\) Three of the 6 isolates had mutation at the initiation codon of pre-S2. In the S coding region, 50% of isolates had mutation at codon 47 (T\(\rightarrow\)K), which was located in the T cell epitope (codons 41–49).\(^8\) As shown in Figure 2, there were 5 “hot-spots” mutations in the core region, which were all located in the B cell epitopes (codons 76–89, 130–135, 146–159) and T cell epitopes (61–85, 81–105, 117–131).\(^9\) Furthermore, 2 isolates showed a stop-codon mutation in the precore region, which abolished HBeAg expression.

Multiple point mutations were found in the X region (Fig. 3). Two regions (codons 87–101 and 127–131) were located in the hypervariable regions. Two isolates were found with a stop codon mutation at the 87th residue in X region, which would result in truncated X protein. No stop-codon mutation was found in the polymerase gene and no shared common mutation was found among the 6 isolates. Codon 613 in the polymerase gene of #15 isolate was glutamine, whereas it was leucine in all other 5 isolates (alignment of the P gene not shown, but sequences of these isolates are available in GenBank).

**Mutations in the enhancer II/core promoter region**

All isolates had identical mutations at 1762 and 1764 nucleotides in the core promoter region and several mutations were found in the negative regulatory element (NRE) and Box α in the enhancer II region (Fig. 4).

**Replicative efficiency of isolates**

The replicative efficiencies of various isolates were compared by transfecting full-length cloned HBV DNA into HepG2 cells under standard condition. The HBV DNA present in intracellular
Expression of HBsAg and HBeAg in the supernatant of transfected cells

The P/N (positive/negative) value of HBsAg and HBeAg expressed in the supernatant of all 6 isolates are shown in Table II. Levels of expression of these viral proteins varied markedly. The low expression of HBeAg was seen in the 2 isolates with a stop-codon mutation in the pre-core region (#15, #AID), whereas only 1 (#15) was of low replication efficiency (Fig. 5). The ratio of scanned signals are: Control:1; #14:1.03; #15:1; #16:2.01; #20:234; #17:102; #AID:71.

Liver carcinogenesis is a multi-step complex process and the mechanisms of which are still not fully understood. In our study, we have limited our aim to examine the role of hepatitis B virus, which is one of the most important etiological factors associated with HCC in China. As early as 1988, based on epidemiological data, Beasley reported that HBsAg carriers with active virus replication and cirrhosis were at especially high risk of developing HCC. Recently, in a molecular-epidemiological study on HBV replication and HCC, the authors reported that though the HBsAg carrier rate was similar in China and Senegal, most Chinese HBV carriers were serum HBV DNA positive at older age, whereas in Senegal, serum HBV DNA were undetectable around 30 years old and 10 times more HCC were found in China than in Senegal. In accordance with this study, Gu et al. reported that free replicating forms of HBV DNA without the coexisting integrated form were detected in 20% of Chinese HCC tumor tissues. Raimondo et al. described that replicative forms of HBV could be detected in the liver tissues of some HCC patients, in the absence of detectable HBV DNA. The authors suggested that a defect in encapsidation or blocked release of HBV might occur. To study the roles of replicating or defective replicating HBV strains involved in the pathogenesis of HCC, we amplified and analyzed the genomic sequences and replicating efficiencies of full-length HBV genomes from 6 hepatocellular carcinoma tissues. To our knowledge, there has been only 1 publication on full-length sequence analysis of HBV in association with replication efficiency from 1 HCC patient. In our present study, we have further shown that 5 of 6 full-length HBV isolates from different Chinese HCC tissues were highly replicative, providing evidence that HBV strains with a high replication efficiency indeed persisted in the cancer tissues of most HCC patients from Chinese HCC patients. It would be interesting to do a comparative study in Senegalese HCC patients to verify the implication of high replication efficiency HBV isolates from Chinese HCC tissues.

When the nucleotide sequences of the 6 HCC-derived HBV isolates were compared to the consensus sequences of 10 HBV adr subtype strains, no single mutation specific for HCC could be identified. Codon 613 in the P gene of the low replicating isolate #15 was glutamine, however, whereas it was leucine in the other 5 highly replicating isolates. Interestingly, codon 613 was also glutamine in the control low replicating strain #62. Because replication is the outcome of interactions between the complete viral genome and host cells, whether low replication efficiency in #15 and #62 was due to the substitution of leucine by glutamine at codon 613, amino acid substitution studies in the same isolate would help to address this issue.
Full-length sequence analysis of the immune epitopes of HBV showed that all 6 isolates had 1 or more mutation in the T helper or B cell epitopes. A total of 57% (4/7) “hot-spot” mutations in the Pre-S/S gene and 80% (4/5) “hot-spot” mutations in the core gene were located in the putative T or B cell epitopes. Similar mutations in these regions were also described in our previous study of HCC\(^\text{c}\) and by others\(^\text{8–14}\) in HCC, acute, chronic or fulminant hepatitis B cases. These mutations could lead to the emergence of immune escape mutants expressing viral proteins that might not be recognized by host immune surveillance against the wild-type HBV. Thus, the presence of these mutants would predispose the host to chronic infections.

High replicative HBV strains possessing “hot-spots” mutations could be important potential candidates for initiating the multi-step process of HCC development. Though HBV is a non-cytopathic virus, high replicating strains could over-express the HBV core and S protein in infected hepatocytes, as these viral proteins are targets for cell-mediated immune injury.\(^\text{15}\) This could cause severe liver damage. Besides, the multi-functional X protein can transactivate a variety of viral and cellular promoter/enhancer elements, including up-regulating tumor necrosis factor-α gene expression in hepatocytes, which could induce intrahepatic inflammatory processes.\(^\text{16}\) High replicative strains could also express more X protein in hepatocytes and cause liver injury indirectly. Mutations in the X protein could increase its transactivating effects and upregulate viral or cellular genes, resulting in hepatocellular growth and liver injury.\(^\text{16,17}\) “Hot-spots” mutations at nucleotides 1764 A→T and 1766 G→A in the core promoter and the precore 28th stop mutation have been described as associated with more severe hepatitis.\(^\text{18}\) In our study, all isolates had at least 1 of these mutations. Besides, mutations in the Pre-S2 initiation codon and the deletion in the pre-S2 region could block the synthesis of middle protein, retention of overexpressed large protein and cause severe injury to hepatocytes.\(^\text{19}\) Recently, a long-term follow-up study of HBV mutants among renal transplant recipients was reported. By comparing HBV strains from patients who developed cirrhosis or end-stage liver disease with those from patients who showed no evidence of cirrhosis, complex HBV populations with mutations in core promoter, core gene and Pre-S region were reported as associated with development of cirrhosis.\(^\text{20}\) Because most of the mutations observed in the HBV isolates from HCC tissues in our study were similar to the mutations associated with cirrhosis, one would speculate that persistent replication of these isolates could lead to cirrhosis and result in HCC.

From the present functional analysis of full-length HBV genome derived from HCC and our previous finding of enhanced replicative efficiency in post-HCC HBV isolates, we propose that HBV strains that emerged with “hot-spots” mutations and still retaining high replication efficiency could be one of the important factors to initiate the multi-step process of HCC in Chinese patients. Though these strains are not prerequisite for the development of HCC, their presence is potentially important. It was reported recently that when high dosage and long term interferon-alpha was given to make mice bearing human HCC xenografts, tumor growth and recurrence was inhibited.\(^\text{21}\) There was replicating form of HBV in the xenografts, the inhibitory effect could also be associated with the antiviral effect of interferon-alpha. Therefore, antiviral therapy at an early stage of HBV infection, would not only contribute to the prevention of HCC by suppressing infection, but could also help to block HCC development in patients with active virus replication by inhibiting the above mechanism.

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