X gene/core promoter deletion mutation: A novel mechanism leading to hepatitis B 'e' antigen-negative chronic hepatitis B

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Abstract. Mutations in the precore and core promoter regions of hepatitis B 'e' antigen (HBeAg) are implicated in HBeAg-negative chronic hepatitis B virus (HBV) infection (CHB). The objective of the current study was to investigate novel mutant patterns that lead to HBeAg-negative CHB. The . PreX-X genomic region from the sera of HBV-infected patients was amplified, and analysis of the sequences displayed a unique deletion region, 234 nucleotides in length, which was observed in 54 clones and named core promoter deletion (CPD). CPD may have an important role in the cause of HBeAg-negative CHB. In addition, a novel deletion mutation in the X gene was observed in patients with CHB. This deletion mutant codes a 76-amino-acid X factor instead of the X protein. In the present study, a new mutation pattern was discovered that may contribute to the cause of HBeAg-negative CHB, and therefore it is worthy of future studies.

Introduction

Hepatitis B virus (HBV), a hepadnavirus, is a well-known cause of acute and chronic hepatitis, liver cirrhosis and hepatocellular carcinoma (HCC). In 2005, ~400 million individuals carried the virus worldwide, of which >250 million resided in Asia (1). The HBV genome is a 3.2-kb circular, partially double-stranded DNA molecule with 4 overlapping open reading frames (ORFs): PreC-C, PreS-S, P, and X-ORF. The expression of core and polymerase polypeptides, the large surface antigen polypeptide, the middle and major surface antigen polypeptides and the HBV X protein (HBx) polypeptide are directed by 4 HBV promoters: Cp, PS1p, Sp and Xp, respectively, and influenced by 2 HBV enhancers, enhancer I and enhancer II (2,3).

Mammalian hepadnaviruses encode the small regulatory protein HBx. This is transcribed from the X gene independently from the other viral transcripts under the control of enhancer I and X promoter. HBx is a small, conserved viral protein that is 154 aa in length with a number of amino acid substitutions, including those at codons 94 (4), 31 (5) and 38 (6). It is a multifunctional regulator that modulates a variety of host processes through directly or indirectly interacting with virus and host proteins (7-9). HBx is a promiscuous transactivator that can activate a variety of viral and cellular promoters and enhancers (10-12). No clear counterpart exists in the nononcogenic family of avian hepatitis viruses. HBx has long been suspected to potentiate hepatocarcinogenesis as HCC has not been observed in avians infected with avian hepadnaviruses in which X-ORF is absent. The X-ORF is partly overlapped by the P ORF at the N-terminal, the PreC ORF and several *cis*-elements of the PreC promoter at the C-terminal. Therefore, mutations in the C-terminal portion may result in mutations of HBx and the *cis*-elements at the same time.

In 1990, Loncarevic *et al* (13) discovered a small ORF upstream of X-ORF, which was designated the pre-X region, that can be expressed in-frame with the X-ORF. Pre-X is 168 nt in length and encodes an additional 56 aa. This implies that the whole of X (PreX-X) encodes a 210-aa protein. A previous study demonstrated that the strain coding the pre-X region is common in China (14). Additionally, a study by Takahashi *et al* (15) suggested that the pre-X region is associated with HCC.

Throughout the course of chronic HBV infection (CHB), the loss of hepatitis B 'e' antigen (HBeAg) and the appearance of antibodies directed against it (anti-HBe) are commonly accompanied by the cessation of viral replication. Such a serological profile may be observed in patients who have precore (PreC) and basal core promoter (BCP) mutants. HBeAg-negative HBV results from the common genomic mutation; G1896A, which converts codon 28 of the precore sequence to a termination codon (TGG>TAG) and thus prevents the expression of HBeAg (16). Another group of mutations affect the BCP region and this results in

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a transcriptional reduction of precore mRNA, but not pregenomic or core mRNA. These HBeAg-suppressive strains contain the mutations A1762T and G1764A in the BCP region and are the predominant quasispecies in patients with chronic hepatitis (17,18). These changes are associated with the HBeAg-negative CHB, but another study demonstrated that these mutants are also found in certain HBeAg-positive patients, particularly those with CHB. This study suggests that HBeAg may be a target antigen on HBV-infected hepatocytes, and the failure to produce HBeAg may be a means of evading immune clearance. Nevertheless, the significance of additional mutations in the precore and core promoter (CP) regions for HBeAg production is unknown (19).

A deletion mutation of the X gene/CP region was previously observed in one patient undergoing HBeAg seroconversion spontaneously, with a length of 234 bp (accession numbers: EF608587-EF608597) (20). In the present study, sequences bearing the PX-X region were investigated, in addition to the enhancer II, the CP, and the precore start codon in order to identify the specific mutations associated with HBeAg-negative CHB.

Materials and methods

Patients. A total of 21 patients were consecutively analyzed: 7 HBeAg-positive and 14 HBeAg-negative; one HBeAg-negative patient was also HB 's' antigen-negative. All patient profiles were maintained by routine testing and confirmed by repeat tests every 3-6 months at least twice. A total of 19 patients were inferred to suffer from CHB and 2 patients were diagnosed with liver cirrhosis. Of the patients, 2 were brothers (X441 and X464), 2 were mother and son (X476 and X475, respectively), and 3 were mother, daughter and son (X461, X462 and X463, respectively). Patients were excluded if they exhibited evidence of autoimmune hepatitis or markers of hepatitis C virus, hepatitis D virus (HDV), or human immunodeficiency virus. Blood samples were collected and the sera stored at -80˚C until use. The present study was conducted in accordance with the Declaration of Helsinki (1964). Approval from the Ethics Committee of Zhongshan Hospital (Xiamen, China), and written informed consent from all participants, was obtained for the current study.

Polymerase chain reaction (PCR). DNA was extracted from 200 μl serum with a QIAamp DNA Mini kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The HBV PreX/X genomic region was amplified by PCR using the sense primer PX1 (5'-CAAGTGTTT GCTGACGCAACC-3', nt 1176-1198) and antisense primer S2 (5'-ACAGCTTGGCGGCTTGAACAG-3', nt 1859-1880), which amplifies the entire HBV PX/X region. PCR assays were performed under the following conditions: 94˚C for 3 min, 35 cycles of 94˚C for 40 sec, 58˚C for 40 sec and 72˚C for 40 sec, followed by a final extension of 72˚C for 10 min. The resulting PCR products (25 μl) were loaded onto a 1% agarose gel and electrophoresed. The DNA band was excised and purified and then ligated with the pMD19 T vector (Takara Bio, Inc., Dalian, China). The recombinant plasmids were transformed into TOP 10 (Tiangen Biotech, Inc., Beijing, China) and screened on ampicillin/Xgal-IPTG plates, then identified by restriction enzyme (Takara Bio, Inc., Dalian, China). digestion. The sequencing reactions were analyzed using the ABI3730 automated sequencer (Invitrogen Life Technologies, Shanghai, China). The sequences reported in the current study have been deposited into GenBank (http://www.ncbi.nlm.nih.gov/genbank/) under the following accession numbers: EU043336-EU043352.

Phylogenetic analysis. Nucleotide and amino acid sequences were aligned using Vector NTI, version 10.0 (InforMax Inc., Bethesda, MD, USA).

Table I. Characteristics of patients, and deletion mutations.

	HBeAg-positive	HBeAg-negative
Total patients	7	14
Age (years, mean ± SD)	37.4±12.3	35.7±16.5
Infection history (years)	8.3±11.4	9.6±15.4
Family history (years)	4	7
Sequencing quantity	27	47
Deletion mutations	20	35
No deletion mutation	7	12

HBeAg, hepatitis B 'e' antigen; SD, standard deviation.

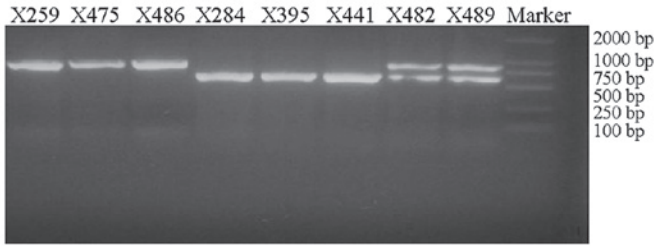

Figure 1. Electrophoresis of the PCR products of target genes on 0.9% agarose gel. bp, base pairs; PCR, polymerase chain reaction.

Results

PCR. The predicted length of the target region, PreX/X or the CP region was 704 bp. Electrophoresis of the PCR products yielded 2 bands following restriction digestion: One that was ~700 bp, and another that was ~500 bp (Fig. 1). This suggests that a 200-bp deletion mutation may reside in the target region. The map also implies that in different patients there are 3 types of PreX/X region: Prototype (X259), deletion mutants (X284) and coexistence of prototype and deletion mutant (X482).

HBV genomic mutations. Using the electrophoresis results, the PCR products of various lengths were ligated into the pMD19 T vector. Subsequent to screening out positive clones, at least 2 clones were selected out of the recombinant plasmid groups from 1 patient for DNA sequencing. All sequences were deposited in the GenBank with the accession numbers EU043336-EU043352. Of these, EU043342, EU043343,

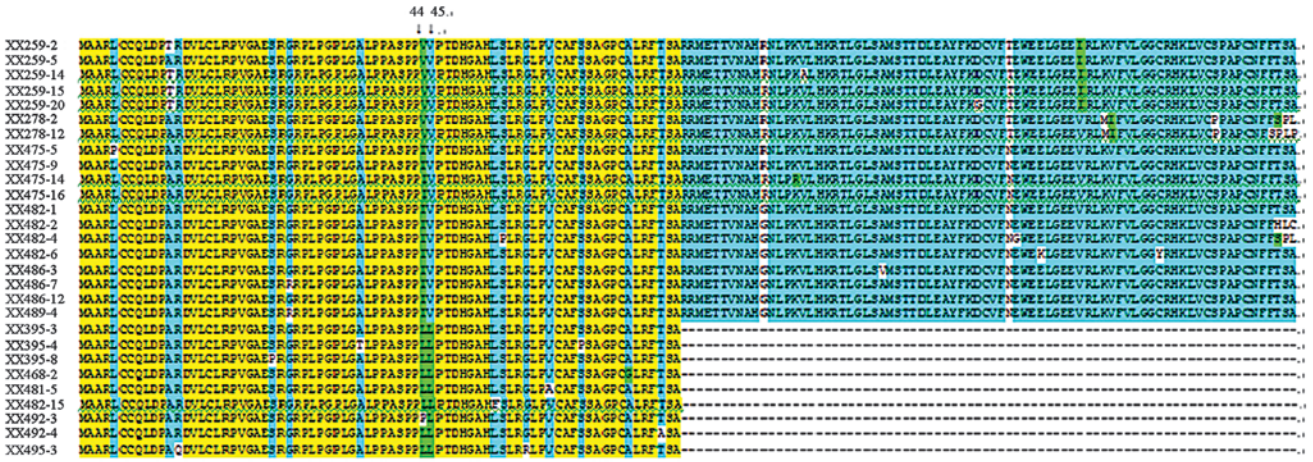

Figure 2. Comparison of the HBx amino acid sequences of the 74 clones. Different truncated coding patterns of HBx are shown. HBx, hepatitis B virus X protein.

EU043344, EU043345, EU043349 and EU043350 had no deletion mutations; EU043351 (XX278-3) had a 245-bp deletion mutation, whilst all others had a 234-bp deletion mutation. None of these clones code for the pre-X region.

In the present study, 74 clones from 21 patients were sequenced (Table I). A total of 54 clones from 19 patients exhibited deletion mutations with a length of 234 nt, at identical positions (nt 1601-1834). The deletion region was found downstream of the X gene (nt 1372-1836) and overlapped the CP (nt 1643-1849). Due to the location of the deletion site, it was named the core promoter deletion (CPD). In addition to the CPD, clone XX278-3 (EU043351) also had an 11-nt deletion, making an overall total of 55 deletion mutations in all patients. The 2 samples X475 and X486, which were HBeAg-negative, had no CPD mutants in their recombinant plasmids groups. Notably, 55 clones had 3 point replacement mutations: G/A1515C, G1518C and A1585T. The sequencing results display that the CPD tandem occurred with the replacement mutations 1585T, 1518C and 1518C, or simultaneously.

In addition, sequences of 6 clones from the brothers, X441 (4) and X464 (2), were identical with CPD. The sequences of the mother X461 (2), daughter X462 (3) and the son X463 (2) were also identical with the CPD. However, there were differences in a number of clones from the mother (X476) and son (X475); 4 clones from the mother were CPD mutants, but none of the 4 clones from the son were.

Out of 19 strains without CPD, 2 had a double A1762T and G1764A nucleotide exchange, XX278-2 and XX278-12, and were from HBeAg-positive patients. A total of 12 strains from HBeAg-negative patients had no such mutation.

HBV protein mutations. The 54 clones bearing CPD encoded a truncated HBx that was only 76 aa in length, so was more like a factor than a protein. There were 9 different truncated coding patterns of HBx in these 54 clones (Figs. 2 and 3). A total of 41 strains from 18 patients coded a characteristic amino acid substitution at amino acids 44 and 45. These CPD strains coded as LL (one is PL), but those without a CPD coded VV/IV. The replacement mutations were either a G/A1515C or G1518C. Strain XX278-3, with a CPD that was 245 nt long, coded no HBx-like protein or factor.

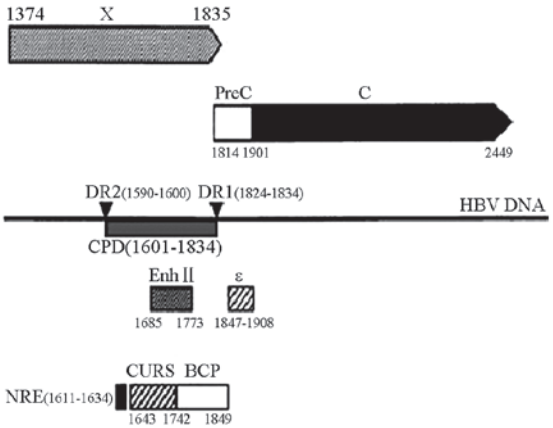

Figure 3. Sketch map of the deletion position in the X gene/core promoter region. CPD, core promoter deletion; HBV, hepatitis B virus; Enh II, enhancer II; NRE, negative regulatory element; BCP, basal core promoter; X, X gene; C, CP.

Discussion

During CHB, two major HBV core gene variants frequently occur that affect the expression of HBeAg: i) The PreC mutants; and ii) the BCP mutants (21,22). In a previous study, the PreC mutants and the BCP mutants from a patient undergoing a spontaneous HBeAg seroconversion were analyzed. In the present study, 20 clones (EF608587-EF608597) with a long deletion mutation in the CP region that was up to 234 nt in length were discovered, which was termed CPD. The CPD not only resulted in deletion of the start codon from enhancer II and preC, but also produced truncated HBx expression. This is a novel mutation pattern resulting in HBeAg-negative CHB. In the present study, samples were collected from 21 patients and the genetic variability of PreX-X/CP region was analyzed. Of them, 7 were HBeAg-positive, and 14 were HBeAg-negative. A total of 55 out of 74 (EU043336-EU043352) strains from 19 patients had a CPD. There was 1 strain with a deletion length of 245 nt, whilst the other 54 strains had a deletion length of 234 nt (the location of the latter deletion was from nt 1601

to 1834) (Fig. 3). In total, 64 strains were discovered from 20 patients bearing an identical CPD mutation position in the HBV genome. One deletion mutant was 11 nt longer than the other 234-nt CPD. CPD prevents the function of the CP, so the preC start codon is absent, and HBeAg is not expressed. In the present study, the HBeAg-negative patients did not have a markedly higher frequency of CPD than that of HBeAg-positive patients. However, CPD mutants were found in strains from all 7 HBeAg-positive patients, but only in 12 out of 14 HBeAg-negative patients. Previous studies have suggested that HBV exists as a quasispecies of wild type and mutant in the HBeAg-positive patients. A total of 7 strains from 2 HBeAg-negative patients have no CPD mutation, nor A1762T and/or G1764A replacement mutation. In the current study, 7 patients from 3 families were included and CPD mutants were only found in 2 of the families. This implies that the CPD mutants could be transmitted between family members. In addition, the CPD mutations occur as 3 point replacement mutations; G/A1515C, G1518C and A1585T. In view of the data produced in the current study, we propose that there is an association between the mutations at nt 1515, 1518 and 1585 and the deletion at nt 1601-1834.

HBx is the only nonstructural regulatory protein of HBV, and has been demonstrated to be associated with the development of liver cancer (23). HBx is a conserved viral protein that is hypothesized, by analogy to woodchuck hepatitis virus X gene, to be required for HBV replication *in vivo* (24). Notably, in the current study it was discovered that the CPD mutants did not encode the 154-aa HBx, but a truncated HBx termed X factor, rather than X protein. Truncated HBx, encoded by CPD mutants, is 76 aa in length, with characteristic amino acid substitution at aa 44 and 45, caused by 2 point replacement mutations at G/A1515C and G1518C. Kumar *et al* (25) demonstrated that aa 58-140 of HBx is the minimum domain required for transactivation function. However, the data from the present study suggests that truncated HBx encoded by CPD mutants may have no capability to transactivate viral or host genes as none of the 74 strains encode the preX region. Therefore, we propose 3 variants of the X-ORF: PreX-X, typical X and truncated HBx coding regions, but further molecular epidemiological studies are required to confirm this.

In the current study sequences, including the entire X region as well as enhancer II, CP and precore/core regions, were investigated in order to identify specific mutations in HBeAg-negative HBV. To the best of our knowledge, this is the first study of CPD mutants in the HBV genome. Using specific PCR primers, CPD mutants were detected in sera of patients. Electrophoresis distinguished 3 different PreX-X/CP regions: wild type, CPD mutants and a mixture of wild type and CPD mutants, which are easily recognizable by the large CP deletion compared with the wild type.

Although molecular epidemiological studies have provided circumstantial evidence for the increased pathogenicity of CP mutants, observations in patients are complicated by variables including individual differences in susceptibility to viral infection or replication, the strength of the immune response, and the coexistence of viral quasispecies. CPD mutation may be a novel molecular pattern consistent with HBeAg-negative CHB. However, neither the CPD mutants nor the PreC or BCP mutants result in the disappearance of HBeAg. This may be due to the presence of a mixed infection with the mutant and wild-type viruses. Further studies are required to identify the prevalence of the CPD mutants in China, and the biological function of truncated HBx (X factor).

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