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A new hepatitis B virus e antigen-negative strain gene used as a reference sequence in an animal model

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ABSTRACT

Infection with hepatitis B virus (HBV) e-antigen (HBeAg)-negative strains is increasingly prevalent. Currently, detailed information of the obtained natural HBV strain is not available except for the B genotype and HBeAg-negative. The aim of the present study was to characterize the natural genetic variation of the HBeAg-negative strain and investigate its function. The genic sequence was determined using Sanger sequencing, and compared to related sequences using alignment and phylogenetic analysis. In vivo, virus-specific serum markers were investigated in CBA/CaJ mice. The sequence had a full genome length of 3215 nucleotides. Sites 122, 125, 127, and 160 in S regions were identified as lysine, threonine, proline, and lysine respectively. The main four point variants including A1762T, G1764A, G1896A, and G1899A were detected in the full-length genome. The genotype of the sequence was B, with subgenotype B2 and serological subtype adw2. The characterize of the natural genetic variation strain showed no reported drug-resistant variant in P region and no reported immune escape site in S region. The strain will increase viral replication and infection for mutations A1762T and G1764A in the basal core promoter region, and mutations G1896A and G1899A in the pre-core region. The G1896A variant resulted in a premature stop codon and abolished HBeAg expression. HBsAg persisted for 26 weeks and HBeAg was still negative in CBA/CaJ mice. The present sequence is representative of the HBeAg-negative genome and may serve as a valuable reference for studying HBeAg-negative strains. The present findings were successfully verified in CBA/CaJ mice, demonstrating good applicability of the sequence.

1. Introduction

The hepatitis B virus (HBV) is a prototype member of viruses in the family *Hepadnaviridae*. HBV infection remains a serious public health concern, causing a multitude of clinical conditions ranging from chronic hepatitis B (CHB) infection, liver cirrhosis (LC), and hepatocellular carcinoma (HCC) [1,2]. There are currently no effective cures for LC and HCC, which are associated with high mortality [3]. Moreover, HBeAg-negative strains have become increasingly prevalent in recent years and difficult to eliminate in patients [4], who are at higher risk of developing HCC [5].

HBV has a small (~3.2 kb) partially double-stranded relaxed-circular DNA genome. The genome contains four open reading frames (ORFs), S, C, P, and X, that encode seven polypeptides respectively: large surface antigen, middle surface antigen, small surface antigen, core antigen, e-antigen (HBeAg), protein P, and HBx [6,7]. HBV has a high replication level of about 10^{12}–10^{13} virus particles per day. The lack of proofreading activity of HBV viral polymerase reverse transcriptase (RT) leads to high genetic variation in HBV genome at any site [8]. HBV has higher variation rates than other DNA viruses [9]. Hence, it is important to establish animal models with DNA genetic backgrounds based on the genome of the HBV source strain.

A natural isolate strain was obtained. We only know it is B genotype and HBeAg-negative. The objective of the present study was to characterize the full-length genome sequence of the natural isolate strain and validate it in CBA/CaJ mice.

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2. Materials and methods

2.1. Sourcing of the strain

A representative sample based on specific selection criteria (genotype B, HBeAg-negative, and male) was selected from 53 patients of the Infection Department, Second Affiliated Hospital of Chongqing Medical University (Chongqing, China), by venipuncture. All procedures related to patient sample collection conformed to the Helsinki Declaration; donors provided written informed consent for inclusion in the study.

2.2. Amplification of HBV DNA

The full-length HBV DNA was amplified by PCR. The designed primers were modified from those used by Li et al. [10] and were synthesized by Invitrogen Bio-Tech (Shanghai, China). The reaction mixture contained 10 μl of 2 × Primestar DNA polymerase (Takara, Dalian, China), 2 μl of DNA, 2 μl of each of the forward and reverse primer, and double-distilled water (ddH_2O) to reach a final volume of 20 μl. The cycling parameters were as follows: pre-denaturation at 95 °C for 2 min; 30 cycles of denaturation at 95 °C for 50 s, annealing at 58 °C for 20 s, and extension at 72 °C for 2 min; and a final extension at 72 °C for 5 min. For verification, the PCR product was digested by EcoRI (Takara, Dalian, China) for 2 h at 37 °C. The reaction mixture contained 1 μl of EcoRI, 1 μl of PCR product, 1 μl of 10 × reaction buffers, and ddH_2O to a final volume of 10 μl.

2.3. Construction of the pEASY-HBV plasmid

The full-length DNA obtained from the strain was cloned into the pEASY-Blunt Simple Cloning vector (TransGen Biotech, Beijing, China) according to the manufacturer's instructions. The pEASY-Blunt cloned plasmid was verified using the BspQI enzyme and sequenced using a direct Sanger sequencer (Invitrogen Bio-Tech, Shanghai, China). The cloning vector was eliminated from the sequencing result, and the origin of the genome was adjusted to the EcoRI restriction site. The sequence was then subjected to a BLAST search for comparison to complete HBV sequences in the National Center for Biotechnology Information (NCBI) database.

2.4. Construction of phylogenetic tree

Phylogenetic analysis was conducted for the complete HBV genome sequences available from the NCBI and HBV (http://hbvdb.ibcp.fr) databases; then, phylogenetic analysis was conducted using the newly derived HBeAg-negative sequence using the Molecular Evolutionary Genetic Analysis (MEGA) software program (version 5). The phylogenetic tree was constructed using neighbor-joining analysis, and reliability was assessed with bootstrap resampling and reconstruction using 1000 replicates. Finally, the genetic distance, geographic location of origin, and genotype of the target sequence were determined from the phylogenetic tree.

2.5. Multiple sequence alignment

To determine the unique genetic background of the HBeAg-negative sequence, it was subjected to multiple sequence alignment using the DNAMAN software program (version 6) for comparison with the reference GenBank sequences JX661478 (genotype B, China), FJ787477 (genotype B, China), D00329 (genotype B, Japan), and D00331 (genotype B, Indonesia). Stable variations were confirmed on the basis of a matching rate $\leq$20%. Nucleic acid and amino acid (aa) sequences of the four partial ORFs (pre-C/C, pre-S/S, P, and X) and the basal core promoter (BCP) region were analyzed separately, with a particular focus on the variable sites. Additionally, the potential relationships between genetic variations identified and the progression of liver diseases were explored based on the literature.

2.6. Production of circularized HBV DNA and establishment of the animal model

A previously reported method was used to generate circularized DNA [4]. Male CBA/CaJ mice (10 weeks of age, specific pathogen-free) were provided by the Laboratory Animal Center of Chongqing Medical University (SCXK (YU) 2017-0001, SYXK(YU) 2017-0023). The mice were maintained according to the institutional guidelines for the care and use of laboratory animals. Twelve CBA/CaJ male mice were divided randomly into three groups. The experimental group (n = 4) was injected with circularized HBeAg-negative HBV DNA using the hydrodynamic method (1 μg/2 mL/mouse, within 5–8 s). The control group (n = 4) was injected with pAAV/HBV1.2 (5 μg/2 mL/mouse). The blank group (n = 4) was injected with normal saline (2 mL/mouse). Serum samples were collected via the tail vein at 0.5, 1, 2, 3, 4, 5, 6, 8, 9, 14, 18, 22, 24, and 26 weeks after injection. All animal procedures were approved by the Ethics Committee of Chongqing Medical University (permit number: 2015019). All surgical procedures were performed using anesthesia to minimize animal suffering.

3. Results

3.1. A natural HBeAg-negative strain

The basic characteristics of the 53 clinical samples, including HBV genotypes. HBsAg and HBeAg status of the isolated strains, are shown in Table S1 of the Supporting Information section. One of the 53 patients had a strain with genotype A, whereas 38 had genotype B (71.7%), and 14 had genotype C. Overall, the patients were predominantly male (88.7%), and 50.9% of the patients were infected with an HBeAg-negative strain. According to the basic characteristics of the 53 clinical samples, a typical sample (number: 11061008) from a 48-year-old man with B type and HBeAg-negative was selected to determine the viral genome.

3.2. Cloning and sequencing

The cloned full-length HBV DNA was sequenced successfully. The adjusted full-length genome is shown in Fig. S1 of the Supporting Information.

3.3. HBV phylogenetic tree

In total, 51 full-length genome sequences (genotypes A–J) were obtained from the NCBI and HBV databases. Forty-one sequences were of genotype B, ranging from subtype B1 to B9. The phylogenetic tree (Fig. 1) showed that the HBeAg-negative strain belongs to genotype B. The sub-genotype was B2 and the strain was determined to have originated from China, and was most closely related to the Chinese strain FJ787477, based on the phylogenetic tree.

3.4. Sequence alignment

The alignment results of the target sequence with the four full-length reference genomes showed that the four overlapping ORFs of the HBeAg-negative sequence were complete. Sixty point variations were identified in the sequence. Significant sequence variations, detected different the HBeAg-negative sequence from D00331, D00329, FJ787477, and JX661478. The entire genome

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