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Influences on hepatitis B virus replication by a naturally occurring mutation in the core gene☆

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Abstract

Little is known about specific naturally occurring mutations of hepatitis B virus (HBV) and underlying mechanisms of their association with fulminant hepatitis. A HBV clone isolated from a patient with fulminant hepatitis was analyzed, and the features of the particular mutations observed around furin cleavage site in core region (A2339G/G2345A) were assessed using an *in vitro* replication model. The clone belonged to genotype B with precore stop codon mutation (G1896A). Replication efficiency of 1.24-fold HBV genome in Huh-7 cells was increased in the presence of A2339G. Further *in vitro* studies using furin inhibitor indicated that the effect of the mutation was probably associated with accumulation of the full-length core protein without cleavage by furin-like protease, suggesting that a processing of the core protein might play an important role in regulation of viral replication. In conclusion, the A2339G mutation was considered as one of the viral factors involved in high replication efficiency.

Keywords: Hepatitis B virus; Core protein; Genotype; Furin; Fulminant hepatitis; Replication

Introduction

Hepatitis B virus (HBV) remains a major human pathogen. Although new infections are preventable through vaccination, new antiviral targets are being sought for the treatment of the estimated 350 million affected individuals worldwide (Lee, 1997). While acute infection with HBV resolves in the great majority of patients, in a proportion of patients HBV can induce fulminant hepatitis or go on to become chronic hepatitis. What factors influence the fulminant or chronic outcome of acute HBV infection are not fully defined. Approximately 10% of adults and 90% of children become persistent HBV carriers after HBV infection, and 1–2 million people die annually as the consequence of infection with the virus, due to liver cirrhosis and hepatocellular carcinoma.

HBV is the prototype strain of the family *Hepadonaviridea*. The virus has an approximately 3.2 kb circular, double-stranded DNA genome with four open reading frames: core (C), polymerase (P), surface (S), and X. Eight genotypes have been detected with a sequence divergence greater than 8% in the entire HBV genome (Okamoto et al., 1988) and been designated by capital alphabet letters from A (HBV/A) to H (HBV/H) in the order of their discovery (Arauz-Ruiz et al., 2002; Norder et al., 1994; Stuyver et al., 2000). The genotypes have distinct geographical distributions and are associated with differing severities of liver disease as well as response to antiviral therapies (Chu and Lok, 2002; Kao, 2002; Miyakawa and Mizokami, 2003).

The core region encodes two gene products, core protein and HBe antigen (HBeAg), translated from two different transcripts of 3.5 and 3.6 kb, respectively; the differences of the two products were; however, only the amino acids at the N-terminus. Most of the synthetic pathway of HBeAg is now clearly established. In 2003, Messageot and colleagues reported

☆ The nucleotide sequence of HBV-DNA isolates used in this study has been deposited in the international DNA database under accession number AB302095.

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that the C-terminal extremity of HBeAg was located at position 154 and that a proprotein convertase, furin, was involved in the maturation (Messageot et al., 2003).

Furin or furin-like protease is a transmembrane proprotein convertase localized in the *trans*-Golgi network (TGN), transported to the plasma membrane and then retrieved back through the endocytic pathway (Molloy et al., 1999). The consensus sequence of the furin cleavage site is RXK/RR, which has strictly required arginine residues at both the head and tail of the cleavage site (Nakayama, 1997). The furin cleavage site of the HBeAg C-terminus was reported as ^{151}RRGR^{154} (Fig. 1) (Messageot et al., 2003). Therefore, their results strongly indicate that the secretory HBeAg ends at Arg^{154}.

Recently, a patient was referred to our hospital for developing fulminant hepatitis B. Sequence analysis of the causal HBV revealed naturally occurring A2339G and G2345A mutations in the core region, and harboring the precore stop codon G1896A mutation. The replication efficiency *in vitro* of these clones was significantly higher than other clones with the G1896A mutation, which had been constructed before (unpublished data). The novel mutations may be responsible for the higher replication efficiency since the region of the mutation sites is adjacent to the furin cleavage site. To elucidate the biological properties of naturally occurring mutations, although the synthetic and secretory pathways of the core protein are not completely understood, we investigated whether the A2339G and/or G2345A mutations influence viral replication following transient transfection into human hepatoma cell lines.

Results

Influence of A2339G on HBV replication

A serum sample was obtained from a patient with fulminant hepatitis, in whom prothrombin time decreased less than 40% of the controls with hepatic encephalopathy of grade II or more within 8 weeks after the onset of the disease. The results of sequence analysis revealed unique mutation, A2339G and G2345A, in core gene. Then, we constructed plasmids with or without mutations and examined virological characteristics (Fig. 1). Huh-7 cells were transfected in 10-cm dish with 5 μg of each plasmid and harvested 2 days posttransfection. Southern blot analysis of core-associated HBV DNA in the cell lysate demonstrated that transfection of both pBj_2339 and pBj_2339/45 showed approximately 1.4-fold increase of core-associated HBV DNA as compared to the pBj_wild construct (Fig. 2A), while the transfection of pBj_2345 construct did not reveal the different result from wild type. The difference between these plasmids resides only in the mutations present in the core gene, as shown in Fig. 1. The transfection efficiency was monitored by reporter plasmids expressing secreted alkaline phosphatase (SEAP). But the correction of the transfection efficiency was not performed because each experiment in this study showed almost equal value. For immunoblot analysis, a particular monoclonal antibody was adopted. Because the anti-core protein monoclonal antibodies (HB50) recognize SPRRR repeats in the arginine-rich domain of core protein, only the full-length core protein, which is not cleaved by furin-like protease, can be detected by the HB50 antibody. Immunoblot analysis of cell lysate revealed approximately 1.3-fold increase of the core protein in pBj_2339 and pBj_2339/45 transfection (Fig. 2A) as compared to pBj_wild and pBj_2345, indicating that the A2339G mutation could be associated with the high expression of the core protein. This was not due to larger amounts of sample of the A2339G mutants, as revealed by reprobing of the same blot with anti-α-tubulin antibodies (lower panel in Figs. 2A and B). Transfection efficiency was monitored by cotransfection with a gene encoding SEAP. These results were confirmed by at least three replicates. Additionally, similar results were also obtained when these clones were used for transfection of HepG2 cells (data not shown).

The A2339G mutation obstructed the function of a cellular proprotein convertase

Precore proprotein undergoes enzymatic maturation by a protease activated both in the post-endoplasmic reticulum compartment and at the cell surface, which is a well known

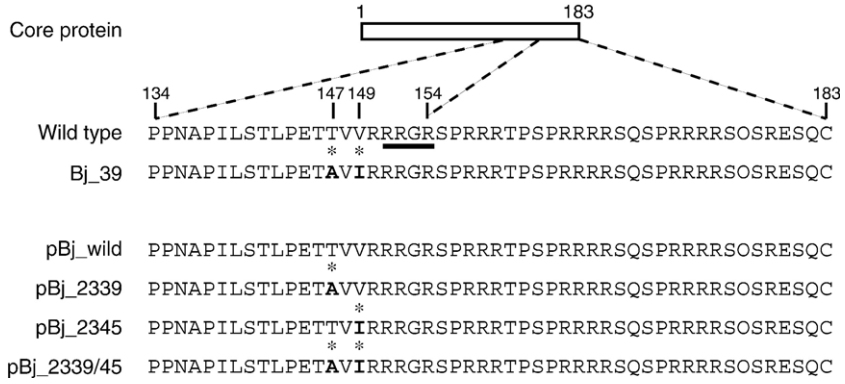

Fig. 1. C-terminus amino acid sequences and substitution mutants of the core protein. The ORF contains two in-frame initiation codons, delimiting the precore sequence and the C gene. Conventionally, position number 1 is assigned to the first amino acid of the core protein. As reported previously by Messageot *et al*., the C-terminus of the precore protein ends at Arg^{154}. The amino acid of the putative furin cleavage site is underlined. The wild-type sequence of the core protein is shown on the upper line. Names of all of the substitution mutants referred to in this paper are on the left. In each case the corresponding sequence is shown, with the mutation indicated in bold and with an asterisk.