Research Article

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Effects of amino acid substitutions in hepatitis B virus surface protein on virion secretion, antigenicity, HBsAg and viral DNA

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Background & Aims: As important virological markers, serum hepatitis B surface antigen (HBsAg) and hepatitis B virus (HBV) DNA levels show large fluctuations among chronic hepatitis B patients. The aim of this study was to reveal the potential impact and mechanisms of amino acid substitutions in small hepatitis B surface proteins (SHBs) on serum HBsAg and HBV DNA levels.

Methods: Serum samples from 230 untreated chronic hepatitis B patients with genotype C HBV were analyzed in terms of HBV DNA levels, serological markers of HBV infection and SHBs sequences. *In vitro* functional analysis of the identified SHBs mutants was performed.

Results: Among 230 SHBs sequences, there were 39 (16.96%) sequences with no mutation detected (wild-type) and 191 (83.04%) with single or multiple mutations. SHBs consist of 226 amino acids, of which 104 (46.02%) had mutations in our study. Some mutations (e.g., sE2G, sL21S, sR24K, sT47A/K, sC69stop (sC69*), sL95W, sL98V, and sG145R) negatively correlated with serum HBsAg levels. HBsAg and HBV DNA levels from this group of patients had a positive correlation ($r = 0.61$, $p < 0.001$). *In vitro* analysis showed that these mutations reduced extracellular HBsAg and HBV DNA levels by restricting virion secretion and antibody binding capacity. Virion secretion could be rescued for sE2G, sC69*, and sG145R by co-expression of wild-type HBsAg.

Conclusion: The serum HBsAg levels were lower in untreated CHB patients with novel SHBs mutations outside the major antigenic region than those without mutations. Underlying mechanisms include impairment of virion secretion and lower binding affinity to antibodies used for HBsAg measurements.

Lay summary: The hepatitis B surface antigen (HBsAg) is a major viral protein of the hepatitis B virus (HBV) secreted into patient blood serum and its quantification value serves as an important marker for the evaluation of chronic HBV infection and antiviral response. We found a few new amino acid substitutions in HBsAg associated with lower serum HBsAg and HBV DNA levels. These different substitutions might impair virion secretion, change the ability of HBsAg to bind to antibodies, or impact HBV replication. These could all result in decreased detectable levels of serum HBsAg. The factors affecting circulating HBsAg level and HBsAg detection are varied and caution is needed when interpreting clinical significance of serum HBsAg levels.

Clinical trial number: NCT01088009.

Introduction

Chronic hepatitis B virus (HBV) infection remains a severe public health problem. About 240 million people worldwide are chronically infected and have an increased risk for developing liver cirrhosis and hepatocellular carcinoma [1,2]. The hepatitis B surface antigen (HBsAg), a major viral protein secreted into patient serum, consists of three distinct, but structurally related proteins: the large, middle and small hepatitis B surface proteins (LHBs, MHBs, and SHBs, respectively). Within the SHBs, the 99 to 169 amino acid (AA) region is termed as the major hydrophilic region (MHR), where AAs 124-147 is defined as the 'a' determinant, a dominant neutralizing epitope [3]. Antibodies used in commercial detection kits usually recognize and bind to this region.

Since its discovery as the Australia antigen by Blumberg *et al.* in 1963 [4], HBsAg has been considered an important biomarker for HBV infection. The loss of HBsAg and the development of anti-HBs antibodies (HBsAg seroconversion) are believed to indicate a clinical cure [1]. The HBsAg quantitative assay is often a better option than the qualitative tests for other biomarkers. [5]. The Architect QT assay (Abbott Laboratories) and the Elecsys HBsAg II Quant assay (Roche Diagnostic) are widely used in the clinic.

Keywords: HBV; HBsAg mutation; Virion secretion; Antigenicity; 'a' determinant; Hepatitis B surface antigens; Amino acid substitution; DNA; Viral; Membrane proteins.

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Both yield quantitative HBsAg (qHBsAg) results, expressed in international units per ml (IU/ml) [6,5].

The wide clinical application of these assays has resulted in the suggestion that the serum qHBsAg might serve as a surrogate marker for transcriptionally active HBV covalently closed circular DNA (cccDNA) in hepatocytces and as a useful marker for guiding antiviral therapy. A study on 26 hepatitis B e antigen (HBeAg)-positive CHB patients receiving a 32-week pegylated interferon (PegIFN) treatment followed by a two-year lamivudine treatment showed that the HBsAg levels correlated with cccDNA ($r = 0.54$, $p = 0.004$) at baseline. Its reduction after treatment showed a good correlation with the reduction of hepatocyte cccDNA levels ($r = 0.68$, $p < 0.0001$) [7]. A study on HBeAg-negative CHB patients showed that a decrease of HBsAg levels by 0.5 $\log_{10}$ IU/ml at week 12 and 1.0 $\log_{10}$ IU/ml at week 24 of PegIFN therapy was associated with the positive predictive values of 89% and 92% for HBV DNA negativity 24 weeks after drug withdrawal, supporting its role as a predictive marker for response-guided therapy in IFN-treated patients [8]. In addition, HBsAg levels could be used as a stopping rule for PegIFN-treated patients [8].

However, significantly different serum HBsAg levels could be found in CHB patients with the same disease progression, HBV genotype and HBeAg status [9]. Locarnini *et al.* showed that serum HBsAg levels only correlated with HBV DNA loads during the immune clearance phase for patients with HBV genotype B and C infection [10,9]. A better understanding of the mechanisms behind serum HBsAg level fluctuation is of clinical importance. Theoretically, the underlying mechanisms could include the factors affecting the HBsAg expression, secretion, antigenicity and/or the immune response. The current study focused on how AA substitutions in SHBs influence qHBsAg values. It is known that new qHBsAg assays have incorporated the impact of some classical SHBs AA substitutions on qHBsAg, such as sK122I, sI126S and sG145R [11]. However, some newly discovered SHBs mutations were under-quantitated by the Architect assay, whereas sP142L, sP142S and sG145K mutations yielded lower results in the Elecsys system when compared with those obtained with the Architect system [12]. In addition, Locarnini *et al.* reported that the rtA181T/sW172stop (sW172*) variant had a secretory defect and exerted a dominant negative effect on wild-type (WT) HBV virion secretion [13].

Beside the aforementioned mutations, SHBs could also have other naturally occurring substitutions affecting serum HBsAg levels, which are yet to be identified or poorly characterized. Thus, the aim of this study was to identify novel mutations that correlated with lower HBsAg levels by using a study cohort of 230 untreated CHB patients, who were all HBeAg-positive and infected with genotype C HBV. The impact and mechanisms of these novel AA substitutions on HBsAg levels were further studied using *in vitro* cell culture systems.

Patients and methods

Patients

Serum samples from 230 untreated HBeAg-positive CHB patients with genotype C HBV were collected from 23 hospitals in China during 2010 to 2012 in a registered clinical study (NCT01088009) [14]. Informed consent was obtained. The clinical diagnosis of CHB was performed according to the Chinese Society of Hepatology [15]. All patients were negative for hepatitis C virus, hepatitis D virus and human immunodeficiency virus serum markers. We selected only genotype C HBeAg-positive patients to exclude the potential impacts of HBV genotype, HBeAg status and disease progression on sequence mutation analysis. In addition, genotype C HBV is the most prevalent genotype in China as wells as some other areas of Asia [16,10,9,17].

PCR amplification, DNA sequencing, and sequence analysis

HBV reverse transcriptase (RT) sequences covering the entire S gene amplification and sequence analysis for identifying the AA substitution in SHBs have been described [18].

Plasmids

Mutants sE2G, sL21S, sR24K, sT47A, sT47K, sC69* sL95W, sL98V, and sG145R were cloned into plasmid pBB4.5 1.2/PC, which contained a 1.2-fold length HBV genome of genotype C with a G1896A mutation in the preC region, to facilitate the DNA replication in the *in vitro* system [19]. Primers for site-directed mutagenesis and overlapping PCR are shown in Supplementary Table 1. Introduction of rtD205H mutation resulted in a polymerase-deficient HBV control plasmid, termed tyrosine-methionine-histidine-aspartate (YMHD), which corresponds to the mutated RT active site tyrosine-methionine-aspartate-aspartate (YMDD). Plasmids with the human influenza hemagglutinin (HA) tag at the C-terminal of SHB derivatives (WT-HA, sE2G-HA, sC69*-HA, sL95W-HA, sL98V-HA, and sG145R-HA) were cloned into pBB4.5 1.2/PC. Since the mutant sC69* has a premature stop codon at the SHBs AA position 69, we inserted a HA-tag at three different SHBs locations (N terminus, before the stop codon between positions 68 and 69, and after the C terminus of WT SHB sequence). A pBluescript II KS (+) plasmid (Addgene, USA) was used as the vector for HBsAg expression. In brief, the preS1, preS2 and S HBV coding regions were inserted into the KpnI and SacI sites of this vector.

Cell cultures and transfection

HepG2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and 1% non-essential AAs (NEAA). HepG2 cells were seeded 24 h before transfection, then cells were transfected with plasmids in the presence of X-tremeGENE 9 at a ratio of plasmids and transfection reagent 1 μg:3 μl (Roche diagnostics, Mannheim, Germany). After overnight incubation, the cells were washed with Dulbecco's phosphate-buffered saline (Life Technologies, USA) five time, and fresh media (DMEM + 10% FBS + 1% NEAA) was added. Transfected cells were harvested and supernatant was collected 72 h post-transfection. Transfection efficiency was assessed by co-transfecting a reporter plasmid expressing enhanced green fluorescence protein. All transfections were performed in triplicate in at least three independent experiments.

Western blot

The protocol for Western blotting has been described [20]. Briefly, HBV-transfected cells from 6 well plates were washed three times with PBS and lysed with RIPA buffer in the presence of a cocktail of proteinase inhibitors (Roche, Mannheim, Germany). Supernatants were loaded on a 4–12% SDS-PAGE gradient gel (Life technologies) and transferred to a polyvinylidene fluoride (PVDF) membrane. Antibodies against HBV S protein: polyclonal horse anti-HBs (cat#: ab9193; Abcam, USA) and monoclonal mouse anti-HBV preS2 (cat#: ab30923; Abcam, USA) and anti-HA-tag (cat#: SAB1411738; Sigma, USA), were used at 1:500 dilutions. SuperSignal® West Femto Maximum Sensitivity Chemiluminescent Substrate (Thermo Scientific, USA) was used for imaging.

Quantification of HBV DNA titers

The qPCR for HBV DNA quantification was performed as previously described [18]. Briefly, serum HBV DNA was quantitated by using the COBAS® AmpliPrep/COBAS® TaqMan® HBV test version 2.0 (Roche Diagnostics, Switzerland). In addition, HBV DNA was extracted by using QIAamp DNA Blood Mini Kit (Qiagen, Germany). HBV DNA in *in vitro* experiments was quantitated by qPCR using the TaqMan Universal PCR Master Mix (Applied Bio systems, Foster) [20]. The primers and probe for qPCR are 5′-CCGTCTGTGCCTTCTCATCTG-3′ (sense), 5′-AGTCCAA GAGTCCTCTTATGTAAGACCTT-3′ (antisense) and 5-/56-FAM/CCGTGTGCA/ZEN/CTTCGCTTCACCTCTGC/3IABkFQ/-3 (probe). The qPCR conditions were: (i) denaturation at 50 °C for 5 min followed by 95 °C for 10 min (one cycle); (ii) qPCR at 95 °C for 15 s, 56 °C for 40 s, and 72 °C for 20 s (40 cycles); (iii) melting at 65 °C for 10 s, followed by 95 °C (continuous).