Research Article

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N-glycosylation mutations within hepatitis B virus surface major hydrophilic region contribute mostly to immune escape

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Background & Aims: HBV immune escape represents a challenge to prevention, diagnosis, and treatment of hepatitis B. Here, we analyzed the molecular and clinical characteristics of HBV immune escape mutants in a Chinese cohort of chronically infected patients.

Methods: Two hundred sixteen patients with HBsAg and anti-HBs were studied, with one hundred eighty-two HBV carriers without anti-HBs as a control group. Recombinant HBsAg bearing the most frequent N-glycosylation mutations were expressed in CHO and HuH7 cells. After confirming N-glycosylation at the most frequent sites (129 and 131), together with inserted mutations, functional analysis were performed to study antigenicity and secretion capacity.

Results: One hundred twenty-three patients had the wild-type HBs gene sequence, 93 patients (43%) had mutants in the major hydrophilic region (MHR), and 47 of the 93 patients had additional N-glycosylation mutations, which were transmitted horizontally to at least 2 patients, one of whom was efficiently vaccinated. The frequency of N-glycosylation mutation in the case group was much higher than that of the control group (47/216 *vs.* 1/182). Compared with wild-type HBsAg, HBsAg mutants reacted weakly with anti-HBs using a chemiluminescent microparticle enzyme immunoassay. Native gel analysis of secreted virion in supernatants of transfected HuH7 cells indicated that mutants had better virion enveloping and secretion capacity than wild-type HBV.

Conclusions: Our results suggest that specific HBsAg MHR N-glycosylation mutations are implicated in HBV immune escape in a high endemic area.

Introduction

After vaccination against hepatitis B virus (HBV) or during the natural history of HBV infection or treatment, the emergence of antibody to hepatitis B virus surface antigen (anti-HBs) is usually associated with protection against HBV or HBsAg clearance; however, many reports have shown that a number of patients have co-existing hepatitis B surface antigen (HBsAg) and anti-HBs [1–8]. This may be the result of selection of immune escape mutants harboring mutations within the HBsAg major hydrophilic region (MHR), which covers amino acids (aa) 99–169 [9–13]. In addition to the classic immune escape mutation (G145R), many other mutations at different positions have been characterized in patients [14–16]. These mutations occur naturally during the course of HBV infection or can be selected under immune pressure [6,17–20].

Some studies have indicated that an additional potential N-glycosylation site (Asn-X-Ser/Thr, where X is any aa except proline) might be created by some mutations within the HBsAg MHR [13,21,22]. Viral envelope N-glycosylation is a post-translational

Keywords: HBsAg; Major hydrophilic region; N-glycosylation; Immune escape; Mutation.

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Abbreviations: HBV, hepatitis B virus; HBsAg, hepatitis B virus surface antigen; anti-HBs, anti-HBs antibodies; MHR, major hydrophilic region; CMIA, chemiluminescent microparticle enzyme immunoassay; AHB, Acute Hepatitis B; CHB, Chronic Hepatitis B; LC, liver cirrhosis; HCC, hepatocellular carcinoma; PCR, Polymerase chain reaction; rHBsAg, recombinant hepatitis B virus surface antigen; ALT, aspartate aminotransferase; aa, amino acid.

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modification that plays an important role in biogenesis, stability, antigenicity, and infectivity in HIV, HCV, and influenza virus [23,24]. Therefore, we hypothesized that additional N-glycan in the HBsAg MHR may play a role in HBV immune escape.

HBsAg prevalence has been significantly reduced in China since the implementation of a universal HBV vaccination program in infants in 1992 [25]; however, several studies have reported the co-existence of HBsAg and anti-HBs in 3% to 5% of chronic hepatitis B (CHB) patients [20,26–28]. The pattern and the characteristics of HBsAg mutations associated with this serological profile remain largely unknown in China.

In the current study we have made a survey of 216 Chinese patients with co-existing HBsAg and anti-HBs, and unexpectedly found that approximately one-half of HBV immune escape variants harbored additional potential N-glycosylation sites. Then we investigated in detail the biological characteristics of the N-glycosylation mutations to gain insight in the role of N-glycosylation mutations in immune escape.

Patients and methods

Study design

Between October 2007 and December 2010, there were 135,847 patients or healthy persons who were tested for HBV serological markers at Ruijin Hospital. Among those tested, 32,467 subjects (24.9%) were HBsAg positive using a chemiluminescent microparticle enzyme immunoassay (CMIA, Abbott, Chicago, IL, USA) and 953 patients (2.9%) had co-existing HBsAg and anti-HBs antibodies. Two hundred forty-eight of the 953 patients (26%) had a HBV DNA level >3.0 $\log_{10}$ copies/ml detected with real-time PCR (PJ Co. Ltd., Shenzhen, China). Of these 248 patients, 216 hepatitis B patients, whose samples were available and had a successful PCR amplification, were enrolled retrospectively. anti-HBs levels <10 IU/ml were considered negative. The diagnosis of acute hepatitis B (AHB) was based on the presence of IgM anti-HBc antibody and ALT level abnormal, spontaneous HBsAg and HBV DNA clearance within 6 months. Chronic hepatitis B was defined as history of HBsAg for more than 6 months, persistent elevation in serum ALT levels or necroinflammation was confirmed by liver biopsy. The diagnostic criteria for inactive HBsAg carrier state was serum HBV DNA <2000 IU/ml (or 4 log copies/ml), HBeAg negative and persistently normal ALT levels, which conform to the 2009 AASLD and 2009 EASL Practice Guidelines for the Management of Chronic Hepatitis B [29,41]. Patients with normal ALT level, HBeAg-positive and serum HBV DNA >20,000 IU/ml are considered to be in the "immune tolerant" phase. Liver cirrhosis and hepatocellular carcinoma (HCC) were diagnosed by imaging tests such as CT scan, MRI and ultrasound, liver biopsy, and/or AFP serology. All but three patients were serum HBsAg-positive and anti-HBs-positive at the first visit. The three patients who were diagnosed as HBsAg+/anti-HBs− at the first visit were shown to be double-positive (HBsAg+/anti-HBs+) during follow-up visits. One hundred eighty-two HBsAg-positive patients with HBV DNA level >3.0 $\log_{10}$ copies/ml (i.e., threshold for PCR amplification and sequencing) without detectable anti-HBs were selected randomly from our hospital and designed as a control group, which involved 67 CHB, 54 liver cirrhosis, 31 immune tolerant and 30 HCC patients. Clinical profiles of the current study population are presented in the Supplementary data.

Polymerase chain reaction amplification, cloning, and sequence analysis

HBV DNA exaction, nested PCR amplification and sequence analysis are described in detail in the Supplementary data. Genotyping was based on identifying aa at positions 122, 127, 160, and 177, as previously reported [29].

Construction of expression plasmids

The plasmid construction methods are described in detail in the Supplementary data.

Cell transfection and preparation of cell lysates

HuH7 and Chinese hamster ovary (CHO) cell culture and transfection were performed as described [30]. The methods are described in detail in the Supplementary data.

PNGase F treatment and western blot analysis of recombinant HBsAg

The methods are described in detail in the Supplementary data.

Antigenicity analysis of recombinant HBsAg

The methods are described in detail in the Supplementary data. Each experiment was repeated 3 times and the mean value was calculated.

Native gel analysis of naked core particles and secreted virion

Virion and naked core particles secreted from transfected cells were separated by a native agarose gel electrophoresis assay. HBV DNA from virions was analyzed by Southern blot hybridization. The methods are described in detail in the Supplementary data.

Statistical analysis

Quantitative variables are expressed as the mean ± standard deviation. Statistical analyses were performed using the Student's t test for quantitative data and the Chi-square test (continuity correction) for qualitative variables; the differences were considered statistically significant at p <0.05.

Results

Patient characteristics

In this cohort of 216 patients with HBsAg- and anti-HBs positive detection, the age ranged from 14 to 78 yr (mean, 40.9 yr). There were 147 males and 69 females. Three patients were HBsAg-positive/anti-HBs-negative on the first detection. Of these three patients, one was an asymptomatic HBV carrier at baseline, but progressed to CHB in the follow-up visit based on a decreased serum HBV DNA (but still detectable) and elevated alanine aminotransferase levels, while the serum anti-HBs converted to positive. The two other patients had received lamivudine or PegIFN-α treatment and had virological breakthrough at the time of HBsAg/anti-HBs double positivity.

Among the entire cohort, the HBV DNA level ranged from 1.85 to 8.97 log10 copies/ml (mean, 5.27 log10 copies/ml). The *HBsAg* gene from 216 patients was amplified and sequenced. Of 216 patients, 209 patients' *HBsAg* gene PCR product were successfully sequenced directly and 7 patients' *HBsAg* gene was clone-sequenced because of unsuccessful sequencing (3 patients) or HBV quasispecies (4 patients). In total 228 sequences from these patients and 182 sequences from the control group were obtained.

Characteristics of aa substitution within the HBsAg MHR of HBV isolates in patients with co-existing HBsAg/anti-HBs

We performed an alignment of 228 deduced HBsAg aa sequences from 216 patients, together with sequences from 182 control sequences and 2 reference sequences from databases. The "N-X-T/S" motif, except the aa 146 site, was identified as an additional potential N-glycosylation mutation site.

Compared with the control group, in different stages of liver disease (Table 1), the frequency of N-glycosylation mutation in the case group was higher than that of the control group (N-glycosylation mutation: 47/216 *vs.* 1/182, p <0.001), while there was no significant difference in the frequency of non-N-glycosylation mutation between the two groups (46/216 *vs.* 27/182, p = 0.10). One hundred twenty-three of the 216