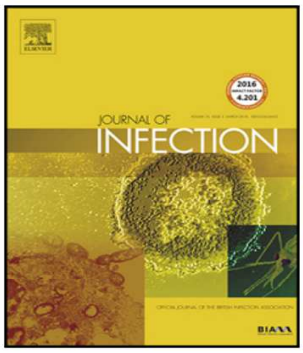
Journal Pre-proof



Construction of an anti-hepatitis B virus preS1 antibody and usefulness of preS1 measurement for chronic hepatitis B patients

Haruna Hatooka , Yumi Shimomura , Michio Imamura , Yuji Teraoka , Kei Morio , Hatsue Fujino , Atsushi Ono , Takashi Nakahara , Eisuke Murakami , Masami Yamauchi , Tomokazu Kawaoka , Grace Naswa Makokha , Daiki Miki , Masataka Tsuge , Akira Hiramatsu , Hiromi Abe-Chayama , C. Nelson Hayes , Hiroshi Aikata , Shinji Tanaka , Kazuaki Chayama

PII: S0163-4453(21)00628-9
DOI: https://doi.org/10.1016/j.jinf.2021.12.025
Reference: YJINF 5368

To appear in: *Journal of Infection*

Accepted date: 13 December 2021

Summary

Objectives: The preS1 region plays an essential role in hepatitis B virus (HBV) infection. We construct an antibody that binds to preS1 and a measurement system for serum preS1 in chronic HBV-infected patients.

Methods: Hybridoma clones that produce anti-preS1 antibodies were obtained by the iliac lymph node method. Epitope mapping was conducted, and an enzyme-linked immunosorbent assay (ELISA)-based method was developed. Using this ELISA system, serum preS1 levels were measured in 200 chronic HBV-infected patients.

Results: Eight types of hybridomas were obtained, of which antibody 3-55 using amino acids 38–47 as the epitope showed high binding affinity to preS1. Serum preS1 levels measured by ELISA using 3-55 antibody were correlated with HBsAg, HBcrAg and HBV DNA levels. Among HBeAg-negative patients without antiviral therapeutic objective (HBV DNA <3.3 log IU/mL or alanine aminotransferase ≤30 U/L), preS1 was significantly higher in subjects who had progressed to the point of requiring antiviral therapy compared to subjects who had maintained their status for the preceding three years ($p<0.01$).

Conclusions: We constructed an antibody against preS1 and an ELISA system capable of measuring serum preS1 levels. PreS1 may serve as a novel tool to predict the need for antiviral therapy in HBeAg-negative HBV-infected patients.

Key words: Hepatitis B virus; Hybridoma; PreS1; Hepatitis B e antigen; Large hepatitis B surface proteins

Introduction

Hepatitis B virus (HBV) is a DNA virus that causes chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC) [1]. The HBV envelope contains three membrane glycoproteins, the large (LHBs), middle (MHBs), and small (SHBs) surface proteins. These proteins are translated from a single open reading frame via different in-frame start codons [2]. SHBs, also known as hepatitis B surface antigen (HBsAg), contains an S domain, MHBs have an extra N-terminal preS2 domain, and LHBs have a preS1 region in addition to the S and preS2 domains. LHBs play an important role in the viral life cycle on both sides of the viral envelope: binding to a virus receptor on the outside and to the nucleocapsid on the inside [3]. HBV cell entry begins with attachment of HBV to the cell membrane via the interaction between viral envelope proteins and heparin sulfate proteoglycans, followed by high-affinity binding of viral envelope proteins to a specific receptor, the bile-acid export pump sodium-taurocholate cotransporting polypeptide (NTCP) [4]. As preS1 is critical for the binding of viral particles to NTCP, LHBs are essential for viral invasion. LHBs could also increase the copy number of covalently closed circular DNA (cccDNA) minichromosomes and initiate replication of cccDNA [5]. The HBV genome encodes two transcriptional activators: the HBx protein and the preS2-activator L protein. Both proteins trigger activation of the c-Raf-a/MEK kinase cascade [6], suggesting that LHBs promotes viral replication. Thus, LHBs seems to be a marker of viral infection and replication.

Following seroconversion of the hepatitis B e antigen (HBeAg) to hepatitis B e antibody (HBeAb), chronic HBV infections may subsequently persist as HBeAg-negative chronic infection or result in HBeAg-negative chronic hepatitis B (CHB) [7]. HBeAg-negative chronic infection is referred to as the inactive carrier (IC) phase, with minimal HBV replication and no inflammation [8]. Recently, it was reported that the composition of HBsAg, LHBs, MHBs and SHBs significantly changes across different stages of HBV infection [9].

In this study, we constructed a monoclonal antibody that effectively binds to HBV preS1, and we developed an enzyme-linked immunosorbent assay (ELISA) system for measuring preS1 antigen levels. Using this ELISA system, we measured serum preS1 antigens in CHB patients and analyzed the relationship between preS1 and the clinical course of HBV.

Materials and Methods

Producing monoclonal antibodies

Genotype C is the most prevalent type of HBV infection in Japan. We designed monoclonal antibodies that react to aa 21–35 (region 1), aa 31–40 (region 2), or aa 37–48 (region 3) regions in preS1 of HBV genotype C (Fig. 1A). Creation of hybridomas and production of monoclonal antibodies that react to preS1 regions 1 or 3 and region 2 were performed by ITM Co., Ltd (Nagano, Japan) and SCRUM Inc. (Tokyo, Japan), respectively. Hybridomas

were obtained by the iliac lymph node method [10]. In brief, mice were immunized with preS1 aa 21–35 peptide for region 1, aa 1–40 and aa 31–60 peptides for region 2, or aa 34–48 and aa 37–55 peptides for region 3. At 2–3 weeks postimmunization, the iliac lymph nodes of the mice were collected, and the lymphocytes obtained from the iliac lymph nodes were fused with myeloma cells. The fusion cells were cultured in 96-well plates, and the activity of the antibody secreted in the culture supernatant was screened. Hybridoma clones that produced the most reactive antibodies for each region were selected.

Sequencing of the variable region of antibody

Total RNA was extracted from each hybridoma cell using the RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA sequencing of immunoglobulin G was accomplished using the 5'-Full RACE Core Set (TaKaRa, Shiga, Japan) according to the manufacturer's instructions. The first cDNA was synthesized with Oligo(dT)12-18 primer phosphorylated on the 5´ end (Thermo Fisher Scientific, Waltham, USA). The first and second PCRs to amplify the immunoglobulin sequences were performed using the primers shown in Supplementary Table 1. The hybridoma cells used in this study expressed not only normal mRNA for the light chain of immunoglobulin G but also junk mRNA corresponding to the same gene. For sequencing of the normal mRNA, PCR primers specific to the normal gene sequence for each hybridoma were designed. Sequencing was performed using BigDye™ Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Sequences of the immunoglobulin were obtained using the 3100-Avant Genetic Analyzer (Thermo Fisher Scientific).

Prediction of the variable region and the complementarity determining region (CDR) were performed on the abYsis website (http://www.abysis.org/) [11] using the Chothia numbering method.

Peptide preparation

Since the selected monoclonal antibodies bound with both peptides 34–48 and 37–55 as described above, the epitopes of the antibodies were considered to be contained within the intersection of the two peptides (NSNNPDWDFNPN), so 8 to 10-mer peptides shifted by one amino acid from the N-terminus to the C-terminus of the common sequence were synthesized by BEX CO.,LTD. (Tokyo, Japan). They were dissolved with nuclease-free water (Thermo Fisher Scientific) or dimethyl sulfoxide to 1 mM and stored at -80°C until use.

Comparison of preS1 detection sensitivity among the monoclonal antibodies

First, the binding ability of the antibody to the preS1 peptide was investigated. Two hundred microliters of 2% glutaraldehyde in 100 mM $NaHCO_3$ buffer (pH 9.6) was added to each well of the 96-well ELISA Plate Amino (Sumitomo Bakelite Co., LTD, Tokyo, Japan)

to activate amino residues on the plate, and then incubated at room temperature (RT) for 2 hrs and washed twice with Milli-Q water. One hundred microliters of 200 nM preS1 peptide (1-68) solution in 100 mM sodium hydrogen carbonate solution (pH 9.6) was added immediately to the wells, and then the plates were incubated overnight at 4˚C. The wells were washed with TBS, 120 µL of ChonBlock Blocking / Sample Dilution Buffer (Chondrex, Redmond, WA, USA) was added as a blocking buffer, and the plates were incubated at 37˚C for 1 hr. After blocking, the wells were washed with PBST (10 mM Na_2HPO_4, 1.8 mM NaH_2PO_4, 140 mM NaCl and 0.05% Tween-20). The antibodies produced by each hybridoma and control antibody (mouse IgG3; M078-3, Medical & Biological Laboratories Co., Ltd. Nagoya, Japan) were diluted to 1 µg/mL, 0.1 µg/mL, 0.01 µg/mL and 0.001 µg/mL with the blocking buffer. One hundred microliters of the antibody dilution were added to the wells in duplicate and incubated at 37˚C for 1 hr. The wells were washed with PBST and then goat anti-mouse IgG H&L, HRP conjugate (Proteintech, IL, USA) was added at a dilution of 1:2000 in ChonBlock Detection Antibody Dilution Buffer (Chondrex). After incubating at 37˚C for 1 hr, each well was washed with PBST, and 100 µL of 3,3',5,5'-tetramethylbenzidine (TMB) solution was added. The wells were then incubated at RT for 15 min. The chromogenic reaction of the TMB catalyzed by HRP was stopped by addition of 100 µL 2N H_2SO_4 solution. The absorbance at 450 nm of each well was measured using Mithras LB940 (Berthold Technologies, Bad Wildbad, Germany).

Subsequently, non-specific binding of antibody to serum was examined. Activation of the amino residues on the ELISA plate was achieved as described above. Immobilization of preS1 on the ELISA plate and subsequent blocking was performed following the same procedure as above. 10% of the diluted sera of a healthy donor and 1.0 µg/mL and 0.1 µg/mL of each of the monoclonal antibodies was mixed and added to wells in triplicate and incubated at RT for 1 hr. The plate was washed with PBST. HRP conjugated Goat anti-mouse IgG (H+L) (Proteintech) were diluted to 1:2000 with the detection antibody dilution buffer. One hundred microliters of each dilution were added to the wells. The plate was incubated at RT for 1 hr and washed with PBST. The detection procedure of preS1 was the same described above. Furthermore, the HBV-detection ability of each antibody was tested. Serum from a patient infected with HBV genotype C with 90000 IU/mL of HBsAg and 8.9 log IU/mL of HBV DNA was used as the standard serum in this study. The HBV preS1 sequence in the serum was the same as that of the peptides used for the mice immunization. The preS1 value in the serum was determined by the ELISA method described below by drawing a calibration curve with recombinant preS1 antigen (Beacle, Inc. Kyoto, Japan). The activated ELISA plate was coated with 2 µg/mL of the monoclonal and control antibodies at 4°C overnight. Each

well of the plate was washed with PBS, 100 µL of the blocking buffer was added, and the plate was incubated at RT for 1 hr. The serum was diluted to 1:250, 1:1000, 1:4000, 1:16000, and 1:64000 with the blocking buffer. Fifty microliters of the diluted serum were added to the wells in duplicate, and the plates were incubated at RT for 1 hr and then washed with PBST. Peroxidase labeled Hyb-824 (IgG3) (Institute of Immunology Co., LTD, Tokyo, Japan) was diluted to 1:500 (approximately 2 µg/mL) with the detection antibody dilution buffer. One hundred microliters of the dilution were added to each well. The plate was incubated at RT for 1 hr and washed with PBST. The detection procedure for HBV binding to anti-preS1 monoclonal antibody was the same as described above.

Epitope mapping

Epitope mapping was performed in triplicate for each antibody secreted by hybridoma clones 3-7, 3-10, 3-13, 3-39 and 3-55. The activation of the amino residues on the ELISA plate was achieved as described above. One hundred microliters of 100 µM peptide solution in 0.2 M phosphate buffer (pH 6.0) was added immediately to the wells and incubated at 4˚C for overnight. The wells were washed with PBS, 200 µL of 5% skim milk in PBST were added as a blocking buffer, and the plate was incubated at 37˚C for 1 hr. After blocking, the wells were washed with PBST. The antibodies secreted by each of the hybridomas were diluted to 1 µg/mL with the blocking buffer. One hundred microliters of the antibody dilutions were added to the wells and incubated at 37˚C for 1 hr. The wells were washed with PBST, followed by addition of the HRP labeled goat anti-mouse IgG (Proteintech) at a dilution of 1:2000 in the blocking buffer, and incubated at 37˚C for 1 hr. After incubation, each well was washed with PBST, 100 µL of TMB solution was added, and the plate was incubated at RT for 15 min. The detection procedure for absorbance was as described above.

Patients

We selected 200 CHB patients who had visited Hiroshima University Hospital between January 2010 and March 2017 and who did not receive antiviral treatment and whose serum samples were available. Of the 200 CHB patients, 23, 29 and 138 patients were infected with genotype A, B, and C HBV, respectively. HBV genotypes in remaining 10 patients were unknown. Twenty-five patients with past HBV infection (negative for HBsAg, positive for HBsAb and/or HBcAb and negative for HBV DNA) and 55 subjects without HBV infection (negative for HBsAg, HBsAb, and HBcAb) were also measured. All participants provided written informed consent for their participation in the study, according to the process approved by the ethics committee of Hiroshima University Hospital (project identification code number No. E-2323) and conforming to the ethical guidelines of the Declaration of Helsinki.

Detection of preS1 in the serum

The detection of preS1 in the serum was performed by the sandwich ELISA method using the antibody secreted by hybridoma clone 3-55 and the commercially available anti-HBsAg antibody, Hyb-824 (IgG3) (Institute of Immunology Co., LTD). After activation of the amino residue on the ELISA plate as described above, the microtiter plate was coated with 2 μg/mL of Hyb-824 diluted in PBS (pH 6.0) at 4°C overnight. Each well of the plate was washed with PBS, 100 μL of the blocking buffer was added, and the plate was incubated at RT for 1 hr. Sera were diluted to 1:10 with buffer. The standard described above was used for standardization of the preS1 values in the sera. The standard serum was diluted to 1:500, followed by two-fold dilutions. One hundred microliters of the serum samples and the standard dilutions were added to the wells, and the plates were incubated at RT for 1 hr. The plate was washed with PBST. Then the peroxidase-labeled IgG of hybridoma clone 3-55, which had been diluted to 1:1000 with the detection antibody dilution buffer, was added to the wells. The plate was incubated at RT for 1 hr and washed with PBST. The detection procedure of preS1 was the same as described above. From the standard curve drawn with the standard serum, the preS1 value of each sample was calculated. For those above the detection sensitivity, they were diluted to 1:100 and 1:1000 and measured again. The detection was performed in duplicate, and the preS1 value was defined as its average. The limits of blank, detection, and quantification for the value of preS1 was determined according to the EP-17 guidelines of the Clinical and Laboratory Standards Institute [12]. The functional sensitivity is defined as 15% of the coefficient of variance. The intra-assay coefficient of variation was 6.75%.

Measurement of HBV markers

HBsAg and HBeAg levels were measured by the chemiluminescence enzyme immunoassay (CLEIA) method using a commercially available enzyme immunoassay kit (Lumipulse, Fujirebio Inc, Tokyo, Japan). HBV DNA level was measured using a real-time PCR assay (COBAS TaqMan HBV Test; Roche Diagnostics, Tokyo, Japan). The detectable ranges for HBV DNA quantitation using these methods is from 1.3 to 8.2 log IU/mL. Serum hepatitis B core-related antigen (HBcrAg) level was measured by a CLEIA HBcrAg assay kit with a fully automated analyzer system (Lumipulse System, Fujirebio Inc). The detection limit for the assay was 3.0 log U/mL.

Statistical analysis

Correlation between preS1 and other markers was investigated using regression models. All variables were compared between the groups using the Spearman rank correlation coefficient test. Cases after HBeAg seroconversion were divided into 4 groups as shown above and tested by the Steel-Dwass method. Furthermore, the Wilcoxon test was performed for comparison between the two

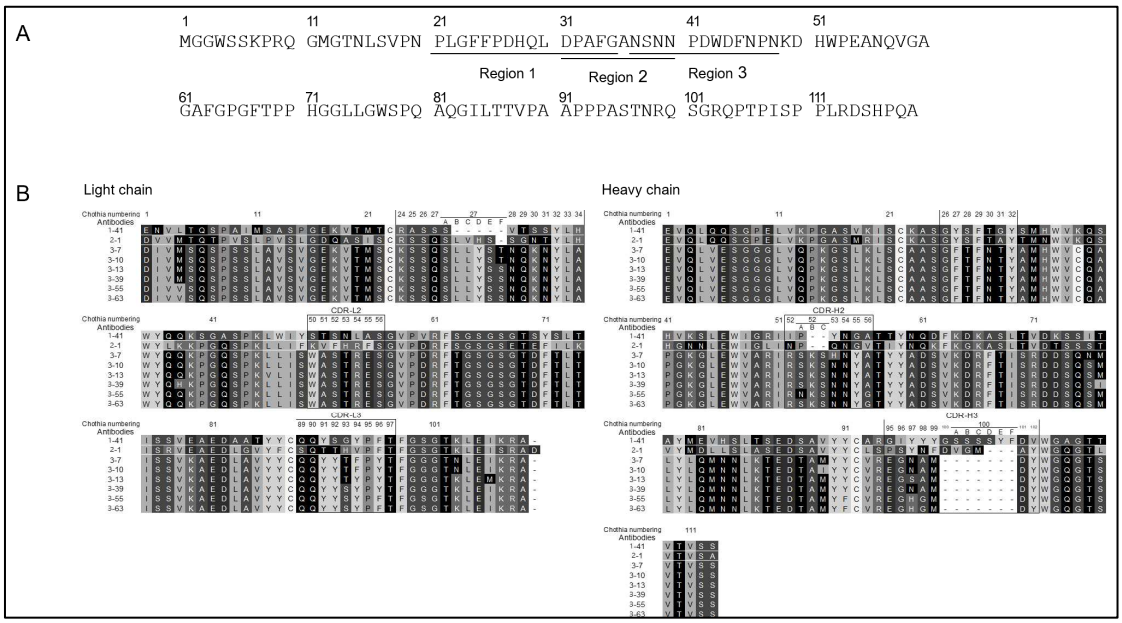


Figure 1. Construction of anti-preS1 antibodies. (A) HBV genotype C preS1 amino acid sequences and the regions involved in immunization in mice. The amino acid sequence of preS1 region of genotype C is shown. In this study, we obtained mouse hybridoma antibodies that react with region 1 (aa 21-35), region 2 (aa 31-40), and region 3 (aa 37- 48) of the 21st to 59th amino acids of preS1. (B) The variable region sequences of the anti-preS1 antibodies. The complementarity determining regions (CDRs) that are expected to be the variable regions of the heavy and light chains of each antibody are shown. The antibodies in region 3 were highly homologous to each other. Within the CDR sequence, one or two amino acids were different between the heavy chain and the light chain for each antibody.

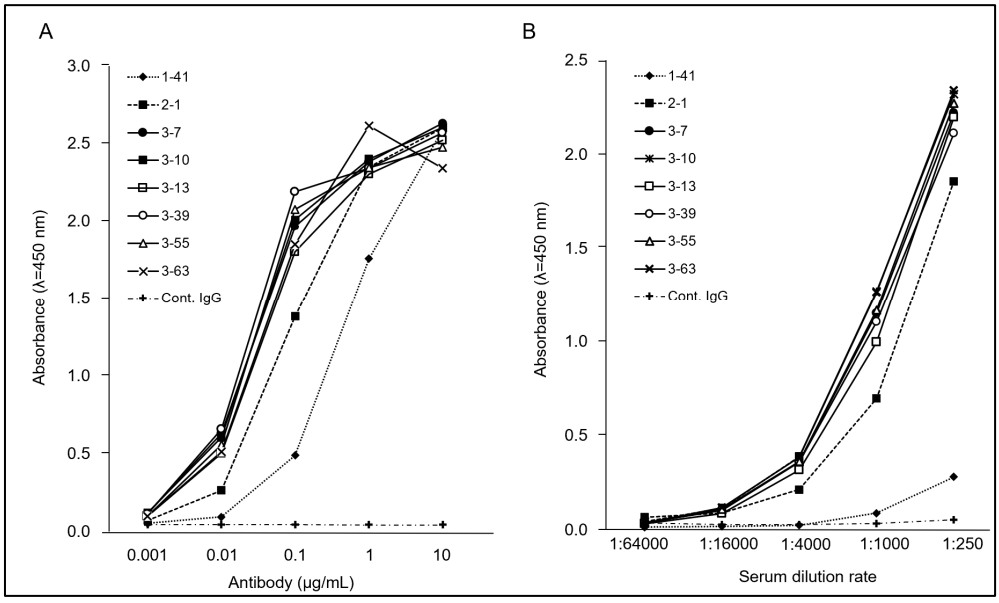


Figure 2. Affinities of constructed anti-preS1 antibodies. (A) Reactivity of the anti-preS1 antibodies to

preS1 peptide. Antibodies that react with regions 1 and 2 are less reactive than antibodies that react with region 3, and a higher concentration of antibody was required to obtain a signal. The reactivity of an antibody that reacts to region 3 to preS1 was similar. Mean values of duplicate measurements are shown. (B) Detectability of preS1 in serum from an HBV patient. The antibody that reacts with region 3 had the highest preS1 detection ability in serum, and all the antibodies showed the same detection ability. 2-1 was slightly inferior to the detection ability of the antibody in region 3, and 1- 41 had the lowest preS1 detection ability. Mean values of duplicate measurements are shown.

groups. Values less than the limit of quantification were replaced by small values (half of the minimum positive value in the original data). All *p* values less than 0.05 were considered significant. Numerical data were calculated using JMP Pro, version 15 (SAS Institute Inc., Cary, NC).

Results

Hybridoma and amino acid sequences of the variable region of antibodies

Eight types of antibodies were obtained: antibody 1-41 reacted to region 1 (preS1 aa 21–35), antibody 2-1 reacted to region 2 (preS1 aa 31–40) and antibodies 3-7, 3-10, 3-13, 3-39, 3-55 and 3-63 reacted to region 3 (preS1 aa 37–48) (Fig. 1A). All of the antibodies were IgG1 and contained a kappa light chain. Amino acid sequences of the variable regions of each hybridoma IgG are shown in Figure 1B. The variable region of the light chain was between aa 21 and aa 135 and that of the heavy chain was aa 20–137. Hybridoma clones 3-7 and 3-10, and clones 3-39 and 3-55 had the same CDR in the light chain, whereas clones 3-10 and 3-39 had the same CDR in the heavy chain. There were no clones that had the same CDR for both the light and heavy chains.

Comparison of preS1 detection sensitivity among the monoclonal antibodies

The reactivity of each antibody with the preS1 peptide was examined. The antibodies that reacted to region 1 and region 2 were less reactive than the antibodies that reacted to region 3, and a higher concentration of antibody was required to obtain a signal. The reactivities of antibodies that reacted to preS1 region 3 were similar (Fig. 2A).

Subsequently, we confirmed the specificity of the antibodies for the preS1 peptide in the presence of human serum. Although there was an antibody whose absorbance was significantly increased in the presence of serum, no apparent decrease was observed, suggesting that nonspecific reaction between the antibody and serum components rarely occurs (Supplemental Fig. 1). Furthermore, the detection ability of each antibody for preS1 in the serum of the highly HBV-infected patients was investigated. The preS1 level in the serum was 240.24 ng/mL, and the concentration of the serum diluted at a ratio of 1:250 was 1.0 ng/mL. Similar to the results of detecting the preS1 peptide by each antibody,

the antibodies reacting with region 3 had the highest detectability of preS1 in the serum, and all the antibodies showed the same detectability. PreS1 detectability of antibody 2-1 reaction to region 2 was slightly inferior, and the detectability of antibody 1-41 was the lowest (Fig. 2B). Based on these results, antibodies against preS1 region 3 seem to have high binding affinity for HBV and react nonspecifically with serum to a lesser extent than antibodies against regions 1 and 2. Therefore, antibodies against region 3 were used in subsequent epitope mapping.

Epitope mapping

ELISA of the N-terminal deletion peptides using the antibody generated by clone 3-13 showed that the antibody bound to the aa 39–47 peptide (NNPDWDFNP) weakly, while it retained full binding activity to the preS1 aa 38–47 peptide (SNNPDWDFNP), indicating that aa 38 is necessary for the antibody binding (Fig. 3). In the case of the C-terminal deletion peptides, the antibody bound to the preS1 aa 38–46 peptide (SNNPDWDFN) weakly, while it retained the full binding activity to aa 38–47 peptide. These results indicate that the epitope of antibody 3-13 is located within aa 38–47 (SNNPDWDFNP) of preS1. For clones 3-39, 3-7, 3-10 and 3-55, epitope mapping was also performed, and we found that the epitope was aa 38–47, the same as in clone 3-13. Although each antibody against region 3 had the same epitope, the antibody productivity of 3-55 was higher than the others, so antibody 3-55 was used in subsequent experiments.

Correlation between preS1 antigen and HBV markers

Using the constructed preS1 antibody, serum preS1 antigen was measured in 200 CHB patients, 25 patients with past HBV infection, and 55 subjects without HBV infection by the sandwich ELISA method. Each R^2 value of the standard curve was 0.99 or more. The limits of blank and detections of the ELISA system was 0.53 and 2.02 pg/mL, respectively, and the limit of quantification was calculated as 4.48 pg/mL. The median value of preS1 in CHB patients was 2.82 log pg/mL, and that of subjects with HBsAg-negative patients and without HBV infection were 1.13 and 1.10 log pg/mL, respectively (Fig. 4A). To identify the cutoff value, a receiver operating characteristic (ROC) curve was created. The area under the curve (AUC) value was 0.89, and the preS1 value at the point closest to the ideal value (0, 1), 1.7 log pg/mL (51.3 pg/mL), was set as the cutoff value. The sensitivity and specificity were 0.79 and 0.93, respectively (Fig. 4B). We next analyzed the relationship between levels of preS1 and HBV markers such as HBsAg, HBeAg, HBcrAg, and HBV DNA in 200 CHB patients. PreS1 values were correlated with all HBV markers, especially with HBsAg (R^2=0.851 and p<0.0001) (Fig. 5). Significant correlation between serum preS1 and HBsAg values was observed in all genotypes, including genotypes A, B, and C (Supplemental Fig. 2). Therefore, subsequent analysis was performed by including all HBV

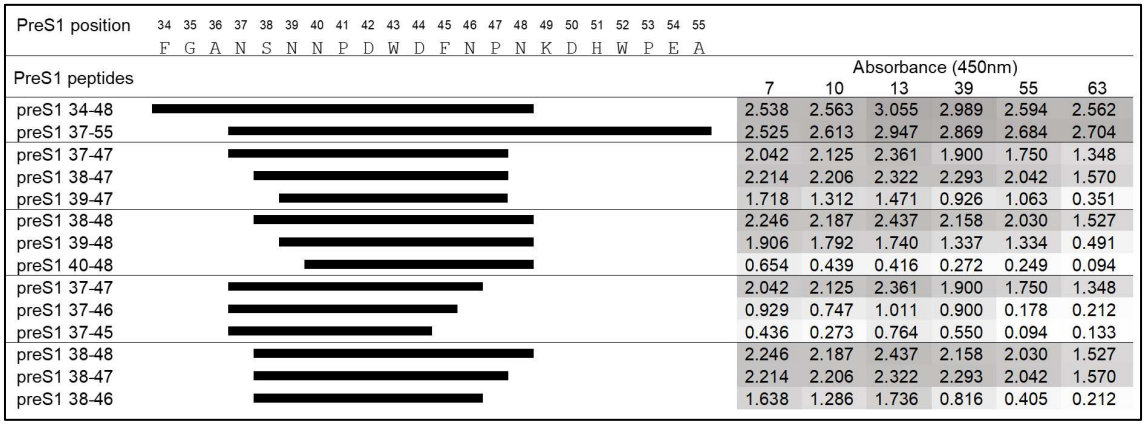


Figure 3. Epitope mapping of region 3 antibodies. The absorbance of the reaction 545 between peptides of various lengths and region 3 antibodies is shown. Peptides of aa 34- 48 and aa 37-55 served as positive controls. Among the amino acids of NSNNPDWDFNPN (37-47) containing the epitope, when the second amino acid from the N-terminal (S) was excluded, the signal of all antibodies decreased. In addition, when the second amino acid from the C-terminal side (P) was excluded, all antibodies showed 550 a remarkable decrease in signal. Since no decrease in signal was observed with SNNPDWDFNP, this sequence was considered to be the epitope of each antibody.

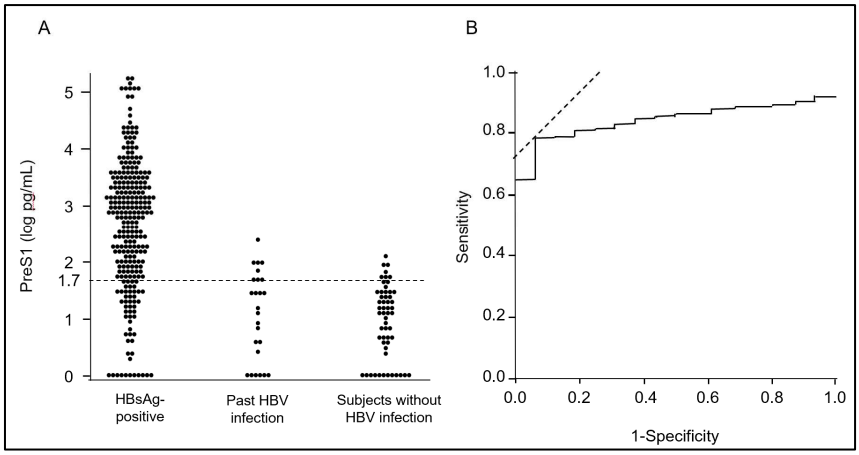


Figure 4. Distribution of preS1 value. A) PreS1 levels were measured in patients with chronic HBV infection (n=200), patients with past HBV infection (n=25), and subjects without HBV infection (n=55). The horizontal dotted line indicates the cutoff value. B) Cutoff value of preS1. To determine the cutoff value, ROC curves were created for 55 subjects without HBV infection and 200 patients with chronic HBs-positive HBV infection. The AUC value was 0.89, indicating moderate accuracy. The preS1 value at the point closest to the ideal value (0, 1), 1.7 log pg/mL (51.3 pg/mL), was set as the cutoff value. At that time, the sensitivity was 0.79 and the specificity was 0.93.

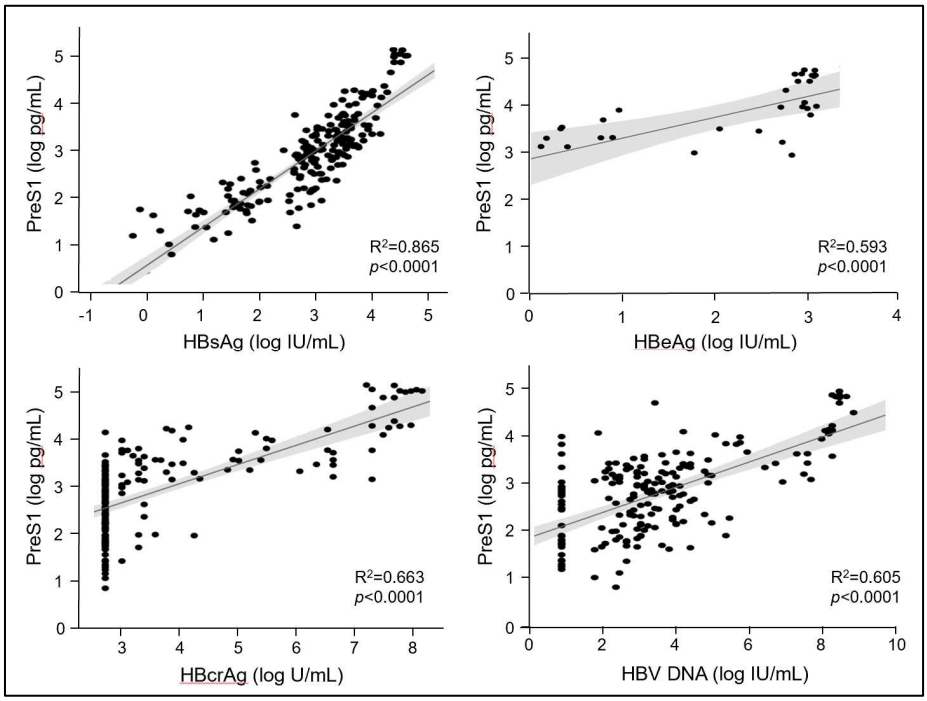


Figure 5. Correlation between preS1 and HBV markers. Correlations between preS1 and HBsAg (n=200), HBeAg (n=32), HBcrAg (n=200) and HBV DNA (n=200) levels were investigated. In the regression model of preS1 and HBsAg, the R^2 value was 0.865 and the p value was <0.0001. suggesting that preS1 level is highly correlated with HBsAg level. A high correlation was found between preS1 and HBcrAg, with R^2=0.663 and p<0.0001. In addition, a correlation was found between preS1 and HBV DNA levels with R^2 =0.605 and p <0.0001.

genotypes.

Characteristics of preS1 in patients with different HBV statuses

The flowchart of the study population is shown in Figure 6. Among the 200 CHB patients, 168 patients were negative, and the remaining 32 patients were positive for HBeAg. Both HBsAg and preS1 levels in HBeAg-positive patients were significantly higher than those in HBeAg-negative patients (p<0.001) (Supplemental Fig. 3). HBeAg-negative CHB patients were classified into four groups depending on HBV DNA and ALT levels: I) HBV DNA <3.3 log IU/mL and ALT ≤30 U/L; II) HBV DNA ≥3.3 log IU/mL and ALT ≤30 U/L; III) HBV DNA <3.3 log IU/mL and ALT >30 U/L; and IV) HBV DNA ≥3.3 log IU/mL and ALT >30 U/L (Table 1). Although the HBsAg values were not significantly different among groups, the value of HBcrAg was significantly higher in group IV than in groups I (p=0.0001) and III (p=0.0154) (Supplemental Fig. 4). The value of preS1 was significantly higher in group IV (p=0.0010) than in group I.

We next analyzed the relationship between the HBsAg and preS1 levels and prognosis of the

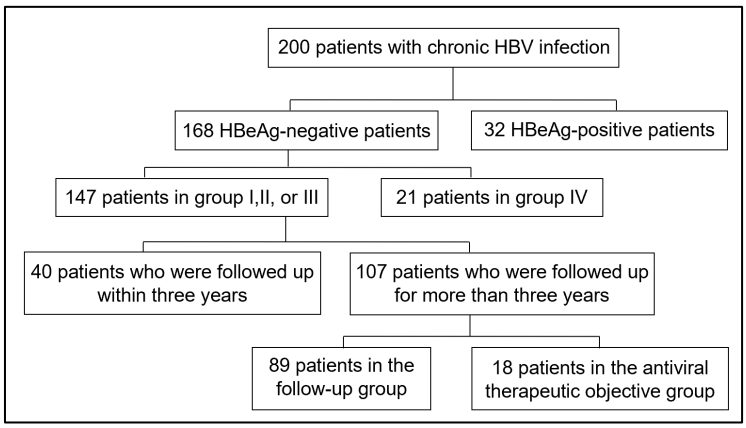


Figure 6. Overview of the study population.

Table 1. Clinical and virological characteristics of each patient group.

HBeAg status	Negative (n=168)				Positive (n=32)
Group	I (n=89)	II (n=39)	III (n=19)	IV (n=21)	
ALT (U/L)	≤30	≤30	>30	>30	
HBV DNA (log IU/mL)	<3.3	≥3.3	<3.3	≥3.3	
Gender (male/female)	37/52	14/25	12/7	15/6	14/18
Age (years)	60 (15-82)	51 (27-72)	51 (27-72)	47 (6-70)	38 (4-68)
ALT (U/L)	17 (4-29)	18 (10-30)	38 (31-73)	44 (31-135)	36 (11-404)
HBV DNA (log IU/mL)	2.3 (0.8-3.2)	3.9 (3.3-5.7)	2.0 (0.8-3.1)	4.3 (3.4-7.6)	8.3 (2.9-9.0)
HBcrAg (log U/mL)	2.7 (2.7-4.4)	2.7 (2.7-5.0)	2.7 (2.7-3.3)	3.3 (2.7-6.5)	7.5 (4.6-8.4)
HBsAg (log IU/mL)	3.2 (-0.1-4.5)	3.2 (1.0-4.4)	3.4 (-0.1-4.5)	3.7 (1.7-4.6)	4.1 (0.1-5.0)
PreS1 (log pg/mL)	2.6 (0.4-4.2)	2.9 (0.4-4.2)	3.0 (0.4-4.0)	3.3 (1.9-4.3)	4.2 (0.4-5.2)

Data are shown as median (range)

progression to the status of antiviral therapeutic objective in HBeAg-negative patients. Of 168 HBeAg-negative patients, 147 patients were in group I, II, or III and remaining 21 patients were in group IV (Fig. 6). Of the patients with group I, II, or III, patients who had remained in group I, II, or III for the subsequent three years were defined as the follow-up group, and patients who progressed to group IV were defined as the antiviral therapeutic objective group. Of 147 patients with group I, II, or III, 40 patients were followed up within three years and 107 patients were followed up for more than three years. Of 107 patients who were followed up for more than three years, 89 patients had remained in group I, II, or III (follow-up group), and the remaining 18 patients progressed to group IV (therapeutic objective group) during the median 3.8 years of follow-up. Although the value of log HBsAg and HBcrAg was similar between the two groups, the level of preS1 was significantly higher in the antiviral therapeutic objective group (p=0.0068) (Fig. 7A). As shown by the ROC curve analysis, preS1 better predicted

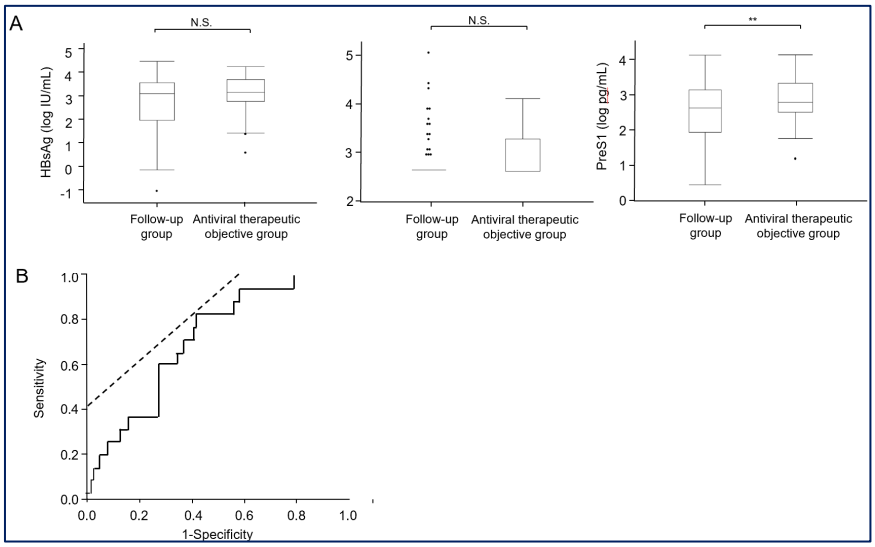


Figure 7. Correlation between preS1 and exacerbation of hepatitis in HBeAg-negative CHB patients. (A) Comparison of HBsAg, HBcrAg and preS1 in the follow-up (n=89) and treatment-indication groups (n=18). In these box-and-whisker plots, lines within the boxes represent median values; the upper and lower lines of the boxes represent the 75th and 25th percentiles, respectively; and the upper and lower bars outside the boxes represent the 90th and 10th percentiles, respectively. Points indicate outliers. (B) Predicted PreS1 value for treatment indication within three years. An ROC curve shows the ability of the linear model to differentiate individuals who had been indicated for HBV eradication in 3 years. The AUC value was 0.723, indicating moderate accuracy. The preS1 value at the point closest to the ideal value (0, 1) was 2.8 log pg/mL. At that time, the sensitivity was 0.83 and the specificity was 0.55. N.S., not significant; **, $p<0.01$

progression to requiring an antiviral treatment within three years among HBeAg-negative CHB patients (Fig. 7B). AUC was 0.723, and when a preS1 cutoff of 2.8 log pg/mL (622.3 pg/mL) was used, the sensitivity was 0.83 and the specificity was 0.55 to predict progression to requiring anti-viral treatment.

Discussion

In this study, monoclonal antibodies specific for preS1 were obtained and subjected to epitope mapping, and a preS1 measurement system by ELISA was constructed. The limit of quantification of this system was 4.48 pg/mL, lower than that of a commercially available system (0.1 ng/mL, Beacle, Inc.). Because genotype C is the most common genotype in Japan, the sequence of genotype C was adopted in this study. We obtained one antibody against region 1 (aa 21–35), one antibody against region 2 (aa 31–40), and 6 antibodies against region 3 (aa 37–48) of the preS1 region, but antibodies against regions 1 and 2 were less sensitive to preS1. As a result of the epitope mapping performed on region 3, the epitope of the antibody was determined to

be aa 38–47 (SNNPDWDFNP). The epitopes of two HBV-neutralizing antibodies, KR359 and KR127, produced by Ryu et al. were aa 19–26 and aa 37–45, respectively, and both antibodies showed a high sensitivity to preS1 [13,14]. The epitope of the antibody against region 3 in the present study was aa 38–47, which was similar to KR127 and was consistent with high sensitivity. In genotype B, the sequence corresponding to the epitope site is SENNPDWDL, which has two amino acid substitutions relative to genotype C, whereas genotype A has the same epitope sequence as genotype C. However, preS1 showed a high correlation with HBsAg in each of the HBV genotypes examined, so analysis was performed using all three genotypes. Jimin et al. reported that the combination of improved antibodies HzKR359-1 and HzKR127-3.2 effectively neutralized genotypes A–D and G–J HBV preS1, so it is thought that different sequences can be bound in spite of these two amino acid substitutions [15]. On the other hand, Peiffer et al. showed that composition of HBsAg differed according to HBV genotype in HBeAg-negative CHB patients [16]. Further analysis is needed to clarify the relationship between the composition of HBsAg and HBV genotype using a larger number of patients.

Previous studies showed that preS1 was correlated with HBsAg, HBeAg, and HBV DNA [17-21]. In this study, preS1 was correlated not only with these HBV markers, but also with HBcrAg. HBcrAg consists of the hepatitis B core antigen, HBeAg, and a 22 kDa pre-core protein encoded by the pre-core/core gene. HBcrAg has been reported as a serum marker that could be used to estimate the extent of the intrahepatic HBV cccDNA pool via production of viral proteins reflecting transcription of messenger RNA from cccDNA [22]. The correlation between preS1 and HBcrAg was consistent with a previous report showing that LHBs plays an important role in increasing the copy number of cccDNA [5].

Cases of high preS1/HBsAg in patients with chronic active hepatitis with high ALT levels have been reported, and it was suggested that this pattern might be associated with liver tissue damage [23], but the actual condition has not been clarified. Distinguishing patients in stable, inactive "carrier" status compared to patients at risk of disease activity or reactivation (IC and CHB) is of paramount importance in clinical practice. In HBV genotype D, Brunetto et al. identified ICs with a sensitivity of 91.1% and a specificity of 95.4% when the patients had HBV DNA <2000 IU/mL and HBsAg <1000 IU/mL [24]. Liu et al. used the same cutoffs in genotypes B and C of Asian cohorts, with sensitivity and specificity of 71% and 85%, respectively [25]. Pfefferkorn et al. reported that although HBsAg levels were similar between IC and CHB, components of HBsAg in LHBs and MHBs were significantly higher in the CHB group in patients with HBsAg >1000 IU/mL [9]. Furthermore, they argued that the components of HBsAg in LHBs and MHBs were more accurate than HBsAg for

predicting IC and were useful markers for distinguishing inactive and active forms of CHB. In the present study, we showed that preS1 was higher in cases with high HBV DNA levels in HBeAg-negative CHB patients. Furthermore, even in cases with low hepatitis activity at the time of measurement, a high preS1 level was associated with a high risk of progression of hepatitis and the requirement of antiviral therapy within the following three years. The cutoff value of preS1 was 2.8 log pg/mL, with sensitivity 0.83 and specificity 0.55, which seems to be a useful marker to predict progression in HBeAg-negative CHB patients.

The reason for the differences in HBsAg composition at various stages of HBV infection is unknown. LHBs transactivates signal transduction and promotes viral replication [3,6] and also plays an important role in increasing the copy number of cccDNA [5]. Therefore, it is estimated that a higher LHBs ratio is associated with a higher virus proliferation ability. In addition, the preS1 region is essential for virus entry and proliferation, as binding to the NTCP receptor occurs within this region [4]. Furthermore, HBsAg is present both in the infectious Dane particles and in noninfectious spherical and filamentous particles. In contrast, LHBs is less detectable in spheres, while it is enriched in filamentous and Dane particles [2]. Thus, it appears that preS1 reflects the amounts of virions more directly than HBsAg. Patients with high HBV proliferative ability have high preS1 levels and are likely to require antiviral therapy. It was reported that the composition of HBsAg provides a better prediction of response to antiviral therapies such as pegylated interferon and nucleos(t)ide analogs, and that reduction of the LHBs ratio during treatment was associated with subsequent HBsAg loss, which reflects stable remission [26, 27]. It would be interesting to investigate the change of preS1 levels in CHB patients who received antiviral therapy using our ELISA system.

In conclusion, we established an ELISA system capable of measuring preS1 levels. Using this ELISA system, we measured preS1 levels in CHB patients and showed that high preS1 is associated with an increased risk of developing CHB. PreS1 levels may serve as a novel tool for predicting progression to the need for antiviral therapy in HBeAg-negative CHB patients.

Acknowledgement

The authors thank Emi Nishio for clerical assistance. This research is partially supported by research funding from the Research Program on Hepatitis from the Japan Agency for Medical Research and Development, AMED (grant numbers: 20fk0310109h0004).

Conflict of Interest Statement

Michio Imamura has received research funding from Bristol-Myers Squibb and AbbVie. Hiroshi Aikata has received honoraria from Eisai and Bayer. Kazuaki Chayama has received honoraria from Bristol-Myers Squibb and MSD K.K., AbbVie, Gilead

Science, Dainippon Sumitomo Pharma and Mitsubishi Tanabe Pharma and research funding from Gilead Science, Dainippon Sumitomo Pharma, MSD K.K., AbbVie, Eisai, TORAY, Otsuka Pharma, Chugai Pharma, Takeda Pharma, and Roche.

Funding statement

This research is partially supported by research funding from the Research Program on Hepatitis from the Japan Agency for Medical Research and Development, AMED (grant numbers: 20fk0310109h0004). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. There was no additional external funding received for this study.

References

1. Ganem D, Prince AM. Hepatitis B virus infection-natural history and clinical consequences. N Engl J Med 2004;350:1118–29. (Pubmed PMID: 15014185 DOI: 10.1056/NEJMra031087)
2. Heermann KH, Goldmann U, Schwartz W, T Seyffarth, H Baumgarten, W H Gerlich. Large surface proteins of hepatitis B virus containing the pre-s sequence. J Virol 1984;52:396–402. (Pubmed PMID: 6492255 PMCID: PMC254539 DOI: 10.1128/JVI.52.2.396-402.1984)
3. Bruss V, Gerhardt E, Vieluf K, G Wunderlich. Functions of the large hepatitis B virus surface protein in viral particle morphogenesis. Intervirology 1996;39:23–31. (Pubmed PMID: 8957666 DOI: 10.1159/000150471)
4. Yah H, Zhong G, Xu G, He W, Jing Z, Gaoet Z, et al. Sodium taurocholate cotransporting polypeptide is a functional receptor for human hepatitis B and D virus. Elife 2012;1:e00049. (Pubmed PMID: 23150796 PMCID: PMC3485615 DOI: 10.7554/eLife.00049)
5. Summers J, Smith PM, Huang M, M S Yu. Morphogeetic and regulatory effects of mutations in the envelope proteins of an avian hepadnavirus. J Virol 1991;65:1310–7. (Pubmed PMID: 1995945 PMCID: PMC239906 DOI: 10.1128/JVI.65.3.1310-1317.1991)
6. Stockl L, Berting A, Malkowski B, Foerste R, Hofschneider PH, Hildt E. Integrity of c-Raf-1/MEK signal transduction cascade is essential for hepatitis B virus gene expression. Oncogene 2003;22:2604–10. (Pubmed PMID: 12730674 DOI: 10.1038/sj.onc.1206320)
7. Fattovich G, Bortolotti F, Donato F. Natural history of chronic hepatitis B: special emphasis on disease progression and prognostic factors. J Hepatol 2008;48:335-52. (Pubmed PMID: 18096267 DOI: 10.1016/j.jhep.2007.11.011)
8. McMahon BJ. Natural history of chronic hepatitis B. Clin Liver Dis 2010;14:381–96. (Pubmed PMID: 20638020 DOI: 10.1016/j.cld.2010.05.007)
9. Pfefferkorn M, Bohm S, Schotto T, Deichsel D, Bremer CM, Schröder K, et al. Quantification of large and middle proteins of hepatitis B virus surface antigen (HBsAg) as a novel tool for the identification of inactive HBV carriers. Gut 2018;67:2045–53.

(Pubmed PMID: 28951526 DOI: 10.1136/gutjnl-2017-313811)

10. Sado Y, Inoue S, Tomono Y, Omori H. Lymphocytes from enlarged iliac lymph nodes as fusion partners for the production of monoclonal antibodies after a single tail base immunization attempt. Acta Histochem. Cytochem 2006;39:89–94. (Pubmed PMID: 17327928 PMCID: PMC1790974 DOI: 10.1267/ahc.06001)
11. Swindells MB, Porter CT, Couch M, Hurst J, Abhinandan KR, Nielsen JH, et al. Integrated antibody sequence and structure-management, analysis, and prediction. J Mol Biol 2017;429:356–64. (Pubmed PMID: 27561707 DOI: 10.1016/j.jmb.2016.08.019)
12. Armbruster DA, Pry T. Limit of Blank, Limit of Detection and Limit of Quantitation. Clin Biochem Rev. 2008;29:S49–52. (Pubmed PMID: 18852857 PMCID: PMC2556583)
13. Ryu CJ, Kim YK, Hur H, et al. Mouse monoclonal antibodies to hepatitis B virus preS1 produced after immunization with recombinant preS1 peptide. Hybridoma 2000;19:185–9. (Pubmed PMID: 10868800 DOI: 10.1089/02724570050031248)
14. Maeng CY, Ryu CJ, Gripon P, Guguen-Guillouzo C, Hong HJ. Fine mapping of virus-neutraizing epitopes on hepatitis B virus PreS1. Virology 2000;270:9–16. (Pubmed PMID: 10772975 DOI: 10.1006/viro.2000.0250)
15. Jimin W, Jeong MS, Hong HJ. Construction and characterization of an anti-hepatitis B virus preS1 humanized antibody that binds to the essential receptor binding site. J Microbiol Biotechnol 2017;27:1336–44. (Pubmed PMID: 28478661 DOI: 10.4014/jmb.1703.03066)
16. Peiffer KH, Kuhnhenn L, Jiang B, Mondorf A, Vermehren J, Knop V, et al. Divergent preS sequences in virion-associated hepatitis B virus genomes and subviral HBV surface antigen particles from HBV e antigen-negative patients. J Infect Dis 2018;218:114–23. (Pubmed PMID: 29528436 DOI: 10.1093/infdis/jiy119)
17. Theilmann L, Klinkert M, Gmelin K, Salfelda J, Schaller H, Pfaff E. Detection of Pre-S1 proteins in serum and liber of HBsAg-positive patients: A new marker for hepatitis B virus infection. Hepatology 1986;6:186–90. (Pubmed PMID: 3957230 DOI: 10.1002/hep.1840060206)
18. Guillou DB, Duclos-Vallee JC, Eberle F, Capel F, Petit MA. Evaluation of an enzyme-linked immunosorbent assay for detection and quantification of hepatitis B virus PreS1 envelope antigen in serum samples: comparison with two commercial assays for monitoring hepatitis B virus DNA. J Viral Hepat 2000;7:387–92. (Pubmed PMID: 10971828 DOI: 10.1046/j.1365-2893.2000.00248.x)
19. Wang NY, Zhang D, Zhao W, Li BA, Lin CQ. Hepatitis B virus large surface protein in serum as a candidate biomarker for evaluating hepatitis B virus infection. Clin Biochem 2011;44:1199–204. (Pubmed PMID: 21782808 DOI: 10.1016/j.clinbiochem.2011.07.002)
20. Zhang A, Li X, Yi W, Li S, Hu C, Chen A. A

monoclonal antibody specific to the non-epitope region of hepatitis B virus preS1 contributes to more effective HBV detection. Clin Biochem 2013;46:1105–10. (Pubmed PMID: 23608352 DOI:25. 10.1016/j.clinbiochem.2013.04.011)

21. Chen L, Hou J, Wei HS, Liu AX, Li PR, Zhao J, et al. Characterization and clinical application of a monoclonal antibody to hepatitis B virus large surface proteins. Frontiers in Laboratory Medicine 2017;1:31–5. (DOI: 10.1016/j.flm.2017.02.007)
22. Wong DK, Tanaka Y, Lai CL, Mizokami M, Fung J, Yuen MF. Hepatitis B virus core-related antigens as markers for monitoring of chronic hepatitis B infection. J Clin Microbiol 2007;45:3942–7. (Pubmed PMID: 17942661 PMCID: PMC2168562 DOI: 10.1128/JCM.00366-07)
23. Chisari FV, Filippi P, Buras J, McLachlan A, Popper H, Pinkert CA, et al. Structural and pathological effects of synthesis of hepatitis B virus large envelope polypeptide in transgenic mice. Proc Natl Acad Sci U S A 1987;84:6909–13. (Pubmed PMID: 3477814 PMCID: PMC299194 DOI: 10.1073/pnas.84.19.6909)
24. Brunetto MR, Oliveri F, Colombatto P, Moriconi F, Ciccorossi P, Coco B, et al. Hepatitis B surface antigen serum levels help to distinguish active from inactive hepatitis B virus genotype D carriers. Gastroenterology 2010;139:483–90. (Pubmed PMID: 20451520 DOI: 10.1053/j.gastro.2010.04.052)
25. Liu J, Yang HI, Lee MH, Jen CL, Batrla-Utermann R, Lu SN, et al. Serum levels of Hepatitis B surface antigen and DNA can predict inactive carriers with low risk of disease progression. Hepatology 2016;64:381–9. (Pubmed PMID: 27079545 DOI: 10.1002/hep.28552)
26. Liu C, Wu W, Shang H, Lin S, Xun Z, Huang E, et al. Prediction value of serum HBV large surface protein in different phases of HBV infection and virological response of chronic hepatitis B patients. Clin Chim Acta 2018;481:12–9. (Pubmed PMID: 29453971 DOI: 10.1016/j.cca.2018.02.015)
27. Pfefferkorn M, Schott T, Böhm S, Deichsel D, Felkel C, Gerlich WH, et al. Composition of HBsAg is predictive of HBsAg loss during treatment in patients with HBeAg-positive chronic hepatitis B. J Hepatol 2021;74:283–92. (Pubmed PMID: 32931877 DOI: 10.1016/j.jhep.2020.08.039)

Construction of an anti-hepatitis B virus preS1 antibody and usefulness of preS1 measurement for chronic hepatitis B patients

Haruna Hatooka , Yumi Shimomura , Michio Imamura , Yuji Teraoka , Kei Morio , Hatsue Fujino , Atsushi Ono , Takashi Nakahara , Eisuke Murakami , Masami Yamauchi , Tomokazu Kawaoka , Grace Naswa Makokha , Daiki Miki , Masataka Tsuge , Akira Hiramatsu , Hiromi Abe-Chayama , C. Nelson Hayes , Hiroshi Aikata , Shinji Tanaka , Kazuaki Chayama

PII: S0163-4453(21)00628-9
DOI: https://doi.org/10.1016/j.jinf.2021.12.025
Reference: YJINF 5368

To appear in: *Journal of Infection*

Accepted date: 13 December 2021

Please cite this article as: Haruna Hatooka , Yumi Shimomura , Michio Imamura , Yuji Teraoka , Kei Morio , Hatsue Fujino , Atsushi Ono , Takashi Nakahara , Eisuke Murakami , Masami Yamauchi , Tomokazu Kawaoka , Grace Naswa Makokha , Daiki Miki , Masataka Tsuge , Akira Hiramatsu , Hiromi Abe-Chayama , C. Nelson Hayes , Hiroshi Aikata , Shinji Tanaka , Kazuaki Chayama , Construction of an anti-hepatitis B virus preS1 antibody and usefulness of preS1 measurement for chronic hepatitis B patients, *Journal of Infection* (2021), doi: https://doi.org/10.1016/j.jinf.2021.12.025