Clinical Biochemistry 47 (2014) 56–62

Contents lists available at ScienceDirect

Clinical Biochemistry

journal homepage: www.elsevier.com/locate/clinbiochem

The differential diagnostic model for serous peptidomics in HBV carriers established by MALDI-TOF-MS analysis

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ARTICLE INFO

Article history:
Received 12 December 2012
Received in revised form 17 October 2013
Accepted 21 October 2013
Available online 29 October 2013

Keywords:
Hepatitis B Virus
Asymptomatic carrier
MALDI-TOF-MS
Fibrinogen beta chain
Nucleophosmin

ABSTRACT

Objectives: Hepatitis B virus (HBV) can result in asymptomatic carrier (AsC) state or chronic inflammation of liver, which depends on the host immunity. We therefore investigated the peptidomic profiling in the process of HBV infection.

Design and methods: In this study, serum from 116 HBV infected (AsC and chronic hepatitis), 60 HBV-immunized and 70 normal subjects was treated with MB-WCX (weak cation exchange based magnetic beads) kits and analyzed by the Clinprot/Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF-MS) techniques. Purified serous proteins were subjected to FT-ICR-MS analysis, and Western blot further confirmed the results.

Results: The specific model comprised of two peptides *m/z* 2882.89 and 4476.12 could distinguish HBV infected from healthy (HBV-immunized and normal) group and showed 95.5% of the sensitivity and 95.4% of the specificity by cross-validation analysis. 40/56 HBV infected and 43/50 healthy subjects could be correctly classified by the model. The area under the receiving operating curves (AUROC) of *m/z* 2882.89 and 4476.12, identified as subunits of fibrinogen beta chain (FBG) Bβ10–42 and nucleophosmin (NPM) respectively, were both up to 0.88 when discriminating AsC from the healthy group. The expression of Bβ10–42 and NPM decreased significantly in the plasma of HBV infected individuals by Western blot analysis.

Conclusions: There were specific serum peptide profilings for host responses to HBV infection, and *m/z* 2882.89 and 4476.12 could be valuable follow-up and prognostic tools for HBV infection.

Introduction

Hepatitis B virus (HBV), a serious healthcare problem, infects approximately 2 billion people globally with 350 million chronic cases [1]. HBV can result in prolonged asymptomatic carrier (AsC) state, acute or chronic hepatitis which can be gradually evolved to cirrhosis and hepatocellular carcinoma [2]. Clinically 75–80% of primary hepatocellular carcinoma (PHC) is intimately related to chronic viral infection and 80–90% is regarded as an end-stage outcome of chronic HBV infection. The host immune response has been implicated in the pathogenesis and clinical outcomes of HBV infection [2,3]. For example, hepatitis B vaccine, enhancing the host immune response, has effectively prevented HBV transmission and the incidence of hepatocellular carcinoma [4–7].

HBV infection and immunity are complicated conditions that involve the changes of cells and molecular mediators in peripheral blood. Now much attention is paid to serological changes of HBV markers such as HBV antigens and genes in the progression of HBV infection [8–10]. Nevertheless, the changes and functions of protein/peptide profilings during HBV infection and immunity are still unknown for us.

In this study, we chose AsC, chronic hepatitis and HBV vaccine immunized subjects to investigate HBV infection-related low molecular weight (LMW) peptides/proteins, and searched for the specific peptides that might influence the differential outcomes of HBV infection and be the predictive indicators for HBV infection.

Experimental

Subjects and materials

The study was approved by the Institutional Review Board of PLA General Hospital and informed consent was obtained from each subject.

Abbreviations: HBV, hepatitis B virus; MALDI-TOF-MS, Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry; AsC, asymptomatic HBV carriers; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; ALB, albumin; FGB, fibrinogen beta chain; NPM, nucleophosmin; ROC, receiver operating characteristic; AUC, the area under the ROC curve; MB-WCX, weak cation exchange based magnetic beads.

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http://dx.doi.org/10.1016/j.clinbiochem.2013.10.016

Serum samples were collected from normal (all HBV related antigens and antibodies negative) and HBV-exposed healthy stuff (HBsAg and HBeAg negative, anti-HBs, anti-HBe, and anti-HBc positive) in PLA General Hospital with no liver disease or risk factor for viral hepatitis and normal liver biochemistry. According to the guidelines from the Chinese Society of Hepatology, the Chinese Society of Infectious Diseases, and the Chinese Medical Association (2005), the hepatitis patients were enrolled from the outpatients with normal US or MRI [11], positive HBsAg and HBV DNA and persistently and/or repeatedly elevated ALT; AsC patients enrolled were HBsAg positive lasting at least 6 months; no previous history of acute hepatitis; no clinical signs or symptoms of liver disease; normal alanine transaminase (ALT), aspartate transaminase (AST), and alkaline phosphatase (ALP) levels on at least two occasions 6 months apart; and no associated non-viral liver disease. A 4 mL blood sample was drawn from each subject, spun and centrifuged; the serum was aliquoted and stored at −80 °C until testing. The entire data set including 116 cases of HBV-infected subjects and 130 cases of healthy subjects was split into 2 sets. The training set (60 cases of HBV-infected, 80 cases of healthy subjects) was used to identify signals and pattern recognition, another blind validation set contained 56 cases of HBV-infected and 50 healthy subjects.

The biochemical parameters including ALP, ALT, AST and albumin (ALB), assayed with commercially available kits (Roche Diagnostic, Penzberg, Germany), were performed to evaluate the liver function. HBV virus markers were quantitatively analyzed by alternative Elecsys HBsAg assay (Roche Diagnostics). HBV DNA was quantitatively analyzed by HBV DNA PCR-Fluorescence Quantitation (KHB Co., Ltd., Shanghai, China).

Serum preparation and MALDI-TOF quantification of peptides

We enriched our low molecular weight protein/peptides by using MB-WCX beads for its superior capture and enrichment ability [12]. Briefly, serum samples (5 μL) were added in 10 μL WCX magnetic beads for separation. The enriched sample was diluted 1:5 in a solution containing HCCA (1.2 mg/L in 2:1 ethanol:acetone), spotted onto the AnchorChip target (Bruker Daltonics Inc., CA) and analyzed on a linear MALDI-TOF mass spectrometer (Microflex; Bruker Daltonics) with the following settings: ion source 1, 20 kV; ion source 2, 18.40 kV; lens, 7.50 kV; pulsed ion extraction, 120 ns; nitrogen pressure, 1700–2000 mbar. Ionization was achieved by irradiation with a nitrogen laser ($\lambda = 337$ nm) operating at 25 Hz. The excess of matrix was initially removed with 10 laser shots at 50% laser power and the acquisition of spectra was carried out at 30% of the maximum laser energy. Each sample was conducted three times. For each MALDI spot, 400 spectra were accumulated in analysis (50 laser shots at 8 different spot positions). Mass calibration was performed after every four samples using a set of peptide/protein standards (No. 206355, 206195, Bruker Daltonics Inc.).

Data processing

To evaluate the reproducibility of the assay, sera from ten normal subjects were pooled and examined 3 peaks to determine the within- and between-run variations. We performed 10 within-run assays and 8 between-run assays to obtain the standard deviation estimate.

Data analysis was performed by Flex analysis, ClinProTools 2.1 software (Bruker Daltonics, GmbH, Bremen, Germany). Comparison of multiple spectra was achieved through the following workflow: all the spectra were recalibrated by using the prominent common *m/z* values; baseline subtraction, smoothing, and peak detection were performed by Flex analysis software. To increase the accuracy, we used the average of eight spots to represent one serum sample. The spectra of all signals with a signal-to-noise (S/N) ratio >5 in a mass range of 1000–10,000 Da were obtained. Since the area enclosed by a peak within its support interval has been suggested as a more useful measure for comparing peaks over a wide range of *m/z* values than the height of a peak [13], we calculated peak areas using end-point level integration type. Basic statistical analysis, Genetic Algorithm (GA), Support Vector Machine (SVM) and Quick Classifier (QC) were used for the selection of signal clusters that were able to differentiate patients originated from different diseases. Diagnostic performance of the signals was evaluated by cross and external validation process. We defined $P < 0.001$ as a significant difference.

Identification of peptide markers

FT-ICR-MS (Bruker Daltonics), equipped with 9.4 T superconducting magnet, nano-HPLC system, and angled off-axis spraying nano-ESI source in the positive ion mode, was used for identification. The voltage of the capillary entrance of the electrospray source was between −1200 and −1600 V. The peptides enriched by magnetic beads were desalted and sequenced in autoMSn mode. CID fragmented the selected ion. All mass spectra were acquired in the broadband mode in the mass range from *m/z* 300 to 3000 with 512 k data points. Agilent ES tuning mix (catalog number G2421A, Palo Alto, CA, USA) was used for calibration. Data were collected and analyzed using software Apex 1.0 and Data Analysis 3.4 (Bruker Daltonics). The MS/MS data were searched on Mascot (www.matrixscience.com). Peptide mass tolerance was 10 ppm, fragment ion mass tolerance was 0.01, and the mass type of parent peptide and peptide fragment was at monoisotopic.

Western blot analysis

Since coagulation/fibrinolysis systems are activated during the blood clotting a large number of fibrinogen and fibrin degradation products (e.g. FpB) are released into the serum, which can affect the detection of *m/z* 2882.89 as a part of fibrinogen beta chain. So we chose plasma in which less interference exists. The peptides were examined by Western blot as previously described [14]. Briefly, plasma pools from AsC ($n = 15$), chronic hepatitis ($n = 15$) and normal ($n = 15$) groups were electrophoresed by SDS-PAGE using 10% (w/v) acrylamide gels and transferred onto PVDF membranes (Amersham). Membranes were probed with the primary and secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The mouse anti-Bβ10–42 antibody (1:10,000) was prepared by Quantobio Biotechnology Co. Ltd, Beijing, China. Rabbit anti-NPM (1:500) was purchased from Sigma-Aldrich (# HPA011384). Densitometric analysis was performed by Bandleader 3.0, and the integrated intensity unit to the peptides was expressed relative to the loading control.

Results

In this study, 116 patients with HBV infection (44 AsC and 72 chronic hepatitis), 130 healthy individuals (60 HBV immunized and 70 normal controls) were enrolled. Details were presented in Table 1. The reproducibility of MALDI-TOF was analyzed by evaluating the within- and between-run of the pooled sample. Three peaks with different molecular masses were selected to evaluate the precision of the assay. CV was calculated and shown in Table 2. The mean CV of within-run assays was 12.0% and that of between-run assays was 19.3%.

The dimensional distribution of the HBV infected (AsC and chronic hepatitis) and healthy (HBV-immunized and normal) groups

We performed the dimensional distribution map of all the samples by MATLAB Principal Component Analysis (PCA). According to the top three principal components, which explained 30%, 24% and 8% of the variance respectively (Fig. 1Panel A), the proteomic characteristics of HBV-infected group were obviously different and could be separated