Clinica Chimica Acta 412 (2011) 2174–2182

Contents lists available at SciVerse ScienceDirect

Clinica Chimica Acta

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

Mass spectrometry-based plasma peptide profiling of acute exacerbation in HBeAg-positive chronic hepatitis B

Eric C. Han [a], Ying-Shiung Lee [a,c], Yu-Ching Liu [a], Hsin-Yi Liao [a], Wen-Sin Liao [c], Hsueh-Chou Lai [b], Cheng-Yuan Peng [b,*], Long-Bin Jeng [d]

[a] Department of Medical Research, China Medical University Hospital, China Medical University, Taichung, Taiwan
[b] Division of Hepatogastroenterology, Department of Internal Medicine, China Medical University Hospital, China Medical University, Taichung, Taiwan
[c] Medical Research Core Facilities Center, Office of Research & Development, China Medical University, Taichung, Taiwan
[d] Department of Surgery, China Medical University Hospital, China Medical University, Taichung, Taiwan

ARTICLE INFO

Article history:
Received 12 December 2010
Received in revised form 30 July 2011
Accepted 1 August 2011
Available online 16 August 2011

Keywords:
Chronic hepatitis B
HBeAg
Acute exacerbation
Magnetic beads
MALDI
Proteomics

ABSTRACT

Background: Acute exacerbations (AE) of serum alanine aminotransferase activities that are 5 times above the normal upper limit frequently occur during the immune clearance phase of hepatitis Be antigen (HBeAg)-positive chronic hepatitis B (CHB). It is unclear how the varying clinical severities of AE reflect differences in the underlying immune responses against the hepatitis B virus.
Methods: We utilized magnetic bead-based purification methods coupled with MALDI-TOF mass spectrometry to generate plasma peptide profiles from HBeAg-positive CHB patients experiencing AE without and with clinical decompensation.
Results: Hydrophobic interaction chromatography (HIC C18) provided a more discriminatory spectral profile than immobilized Cu^{2+} metal ion affinity chromatography did for diagnosis of a clinical spectrum of AEs. Using the sorting algorithm, Support Vector Machine, a classification model consisting of 5 classifiers was determined to give a sensitivity of 94.7% and a specificity of 75% for differentiating patients with and without decompensation. Classifiers identified as fragments derived from transthyretin and apolipoprotein A-IV were significantly decreased and increased in patients with decompensation, respectively.
Conclusions: Our study demonstrated that HIC C18 fractionation coupled with MALDI-TOF mass spectrometry can be used for differentiating AE with and without decompensation in patients with HBeAg-positive CHB.

1. Introduction

Chronic hepatitis B (CHB) is a global health concern with an estimated prevalence of 350 million carriers worldwide. The natural course of CHB infection can be divided into 4 phases: immune tolerance, immune clearance, low or no replication, and reactivation [1]. During the immune clearance phase of the disease, the primary presenting feature is recurrent fluctuations in the activities of serum alanine aminotransferase (ALT). It is common to encounter episodes of abrupt ALT elevations that are >5 times above the upper limit of normal (ULN), which have been defined as states of acute exacerbation (AE) by previous studies [2,3]. Patients presenting with AE have a higher chance of undergoing subsequent hepatitis Be antigen (HBeAg) seroconversion [3]. The severity of AE varies from an absence of symptoms to clinical decompensation with the development of jaundice, coagulopathy, or even hepatic encephalopathy [2–4]. In rare cases, AE leads to fatality [4]. Although AE represents the host's immune effort to clear the replicating hepatitis B virus (HBV), how the different subsets of HBV-specific T cells, such as $CD4^+$ and $CD8^+$ T cells, and HBV-nonspecific inflammatory cells interact to bring about the varying degrees of hepatic necroinflammation remains poorly understood [5–7].

Analysis of proteomic patterns by mass spectrometry (MS) is a relatively novel approach for the identification of potential biomarkers associated with various diseases [8]. Surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS) has been previously applied to predict hepatic fibrosis or cirrhosis in patients with CHB [9,10] and to detect the progression of hepatocellular carcinoma (HCC) in patients with chronic hepatitis C (CHC) [11]. More recently, magnetic bead-based affinity purification with subsequent matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

Abbreviations: AE, acute exacerbation; APOA4, apolipoprotein A-IV; APP, acute-phase proteins; CHB, chronic hepatitis B; CHC, chronic hepatitis C; FGA, fibrinogen α chain; FGB, fibrinogen β chain; HCCA, alpha-cyano-4-hydroxycinnamic acid; HNF, hepatocyte nuclear factor; KNG1, kininogen-1; LIFT, laser-induced fragmentation technology; HIC, hydrophobic interaction chromatography; IMAC, immobilized metal ion affinity chromatography; SOM, self-organizing map; TFA, trifluoacetic acid; TTR, transthyretin.

* Corresponding author at: Division of Hepatogastroenterology, Department of Internal Medicine, China Medical University Hospital, China Medical University, No. 2, Yuh-Der Rd., Taichung 404, Taiwan. Tel.: +886 4 22052121x2264; fax: +886 4 22071600.
E-mail address: cypeng@mail.cmuh.org.tw (C.-Y. Peng).

0009-8981/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.cca.2011.08.011

(MALDI-TOF MS) was introduced for proteomic profiling because of its high sensitivity, reproducibility, and resolution [12,13]. In addition, it has been used successfully to identify potential serum or plasma biomarkers for brain tumors [13], oral cancer [14], and gastric cancer [15]. As a first step in understanding the global plasma proteomic profiles during AE in patients with HBeAg-positive CHB, we utilized magnetic bead-based purification coupled with high throughput MALDI-TOF MS to investigate the plasma peptide profiles of these CHB patients. In particular, we enrolled patients whose conditions were complicated by clinical decompensation. Our aim was to generate patient-specific plasma peptide profiles and to identify unique peptide markers associated with differences in the clinical severity of AE.

2. Materials and methods

2.1. Materials

Matrix: alpha-cyano-4-hydroxycinnamic acid (HCCA), purification kits: MB-HIC C18 (hydrophobic interaction chromatography) and MB-IMAC Cu (immobilized Cu^{2+} metal ion affinity chromatography), and an AnchorChip MALDI target plate (600 μm diameter) were purchased from Bruker (Leipzig, Germany). Peptide and protein calibration standards with average molecular masses ($z=1$) given in parentheses were purchased from Bruker Daltonics: bradykinin (757.86 Da), angiotensin II (1047.19 Da), angiotensin I (1297.48 Da), substance P (1348.64 Da), bombesin (1620.86 Da), rennin substrate (1760.03 Da), ACTH clip 1–17 (2094.42 Da), ACTH clip 18–39 (2466.68 Da), somatostatin 28 (3149.57 Da), insulin (5734.52 Da), cytochrome C $[M+2H]^{2+}$ (6181.05 Da), myoglobin $[M+2H]^{2+}$ (8476.66 Da), ubiquitin (8565.76 Da), cytochrome C $[M]^{+}$ (12360.97 Da) and myoglobin $[M]^{+}$ (16,952.31 Da). HPLC grade water, acetonitrile, ethanol, acetone, and trifluoacetic acid (TFA) were obtained from J. T. Baker (Phillipsburg, NJ).

2.2. Patients and collection of blood samples

Patients with HBeAg-positive CHB who visited the Division of Hepatogastroenterology at the China Medical University Hospital between January 2008 and December 2008 were enrolled in the study. Fifteen healthy adults were invited to participate in the control group (Group 1). Three groups (Groups 2–4) of patients with HBeAg-positive CHB (15 patients in each group) were enrolled and were classified according to the clinical conditions they suffered at the time of blood sampling. The time interval between clinical presentation and blood sampling was 1–2 weeks in all cases. All the blood sampling was taken before the patients received oral antiviral therapy for HBV. All patients in Groups 3 and 4 received lamivudine therapy. Group 2 was comprised of patients with normal serum ALT activities (ULN: 40 IU/L). Group 3 was comprised of patients with AE (serum ALT levels>200 IU/L). Group 4 was comprised of patients with AE and clinical decompensation, which was defined as a prolongation of prothrombin time (PT) ≥3 s longer than the control value and with a total serum bilirubin level ≥2 mg/dl. The serum HBV DNA was measured using the COBAS AMPLICOR HBV Monitor test (Roche Diagnostics, Branchburg, NJ; lower limit of detection: 312 copies/mL). The demographic and clinical characteristics of the healthy controls and the patients are described in Table 1. None of the patients showed clinical or ultrasonographic evidence of liver cirrhosis. The venous blood samples from these 60 individuals were collected after obtaining written informed consent. Approximately 8 mL of each sample was collected in a 10 mL glass tube containing 17.55 mg EDTA. The blood samples were spun at 3500 rpm for 15 min at 4 °C and the plasma portion was immediately aliquoted and frozen for storage at −80 °C until use. The study protocol was approved by the Institutional Review Board of the hospital and was in accordance with the most recent Declaration of Helsinki in 2008.

2.3. Fractionation of plasma proteins

The proteins and peptides in the plasma were purified either by MB-HIC C18 or MB-IMAC Cu fractionation before analysis by MALDI-TOF MS with a Bruker's Ultraflex III MALDI-TOF/TOF instrument. The fractionation of the plasma proteins was performed using ClinProt purification kits, and all of the procedures were performed according to the manufacturer's protocol from Bruker. The detailed experimental procedures for fractionation of plasma proteins are provided in the Supplementary Materials.

2.4. Mass spectrometric measurement of samples

For the preparation of peptides and small proteins on the AnchorChip target plate, 1 μl of bound proteins fractionated by either MB-HIC C18 or MB-IMAC Cu was mixed with 10 μl of matrix solution (0.6 g/l of HCCA in a 1:2 mixture of acetone and ethanol). One μl of this mixture containing the sample and matrix was spotted in triplicate on a 600/384 spot AnchorChip plate with a transponder plate and was left to dry. External calibration was accomplished by applying 0.5 μl of a solution composed of the premixed peptide and protein calibration standards from Bruker and the same matrix, with these spots being targeted in proximity to the spots of the plasma samples. For all mass spectrometric experiments, flexControl 3.0 software was used for data acquisition. A smart laser, Nd:YAG (Gaussian), at 355 nm operating at a repetition rate of up to 200 Hz was used to desorb and ionize the matrix and the sample into the gas phase. To increase the sensitivity of detection, 5 pulses at a laser power of 40% were performed to remove excess matrix prior to acquiring the spectra with 250 pulses at a fixed laser power of 40% from 5 different positions on one anchor point. To generate the peptide spectral profiles, the instrument settings for MS were set as follows: lens at 6.5 kV, nitrogen pressure at 2000 mbar and polarity in the positive mode. All spectra were detected in the linear mode. The accelerating voltage of ion sources 1 and 2 were set to 25.0 kV and 23.2 kV, respectively. The time for pulsed ion extraction was set to 110 ns. The matrix suppression was set up to 700 Da at a high gating strength and only data with a signal-to-noise (S/N) ratio>3 in the m/z range between 800 and 10,000 were acquired automatically using the AutoeXecute tool of the flexControl 3.0 software.

2.5. Data processing and statistical analysis

ClinProTools™ 2.0 software (Bruker Daltonics) was used not only for spectral post-processing, such as baseline subtraction, spectral recalibration, normalization, spectral area calculation, and peak selection, but also for model generation as suggested by the user manual for the software. The detailed parameter settings in ClinProTools™ 2.0 software for the study are provided in the Supplementary Materials. Only data with an S/N ratio>5 in the m/z range between 1000 and 10,000 were acquired and converted into the ASCII file format by ClinProTools™ 2.0. With the assistance of a variety of statistical methods available in ClinProTools™ 2.0 software, the peaks with a $P<0.05$ from the Wilcoxon or Kruskal–Wallis test were considered as statistically significant to discriminate two or ≥2 groups, respectively. With the adjustment of Bonferroni method, the peaks with a $P<0.05$ were regarded as highly significant. To generate the signal pattern of peptide ions visualized by TreeView software (ver 1.6), spectra with ratios of their average peak areas to those of the control group were used for cluster analysis by the two following algorithms: a self-organizing map (SOM) and a hierarchical clustering method [16]. Quantitative comparison of the ion signals in HBeAg-positive CHB patients was determined by the ratio of the mean of spectral areas in the patients to that of the healthy controls and was converted to a binary logarithm for cluster analysis. To identify the peaks with high correlation, the correlation analysis software embedded in ClinProTools™ 2.0 was used to analyze stochastic