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Serum peptide profiles during progression of chronic hepatitis B virus infection to liver failure

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SUMMARY. Chronic hepatitis B virus (HBV)-infected patients with liver failure have a poor prognosis, and no satisfactory biomarkers are available for diagnosis before the end-stage. We explored serum peptide profiling for diagnosis and prediction of progression to liver failure in HBV-infected patients. Serum samples (164) from healthy subjects (n = 20), or subjects with chronic hepatitis B without cirrhosis and liver failure [chronic hepatitis B subjects without cirrhosis and liver failure (CHB); n = 33], with compensated liver cirrhosis (compensated liver cirrhosis (LC); n = 35), with acute-on-chronic liver failure [acute-on-chronic liver failure (ACLF); n = 38] or with chronic liver failure [chronic liver failure (CLF), n = 38] were applied to ClinProt magnetic beads, and bound peptides/proteins were analyzed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. Our classification diagnostic models of liver disease were generated based on the Genetic Algorithm (GA) and Quick Classifier Algorithm (QC). Differentially expressed peptides were found among all test groups, with patterns of difference that readily distinguished between healthy and various HBV-associated liver disease samples. The model generated seven characteristic peptide peaks at 4053 m/z, 3506 m/z, 4963 m/z, 9289 m/z, 2628 m/z, 3193 m/z and 6432 m/z, giving overall predictive capability of 54.27%. Two-way comparisons of LC, ACLF or CLF *vs* CHB had predictive capabilities of 79.8%, 91.41% and 97.99%, respectively. Comparisons of ACLF or CLF *vs* LC were predictive at 87.72% and 82.18%, respectively and ACLF *vs* CLF was predictive at 75.05%. These classification diagnostic models generated by different peptide peaks were further validated in blinded tests with 67–100% accuracy. Serum peptide patterns vary during progression of chronic HBV infection to liver failure and may be used to distinguish different stages of the disease.

Keywords: hepatitis B virus, liver failure, magnetic beads, peptide, serum.

INTRODUCTION

Hepatitis B virus (HBV) infection remains a major health problem worldwide, with approximately 350 million individuals infected. Individuals who are chronically infected with HBV are at risk of developing liver cirrhosis, hepatocellular carcinoma and liver failure. The prognosis of liver failure is very poor, with high mortality [1–5]. Liver failure can develop as acute liver failure, acute-on- chronic liver failure (ACLF), or chronic liver failure (CLF) [6–8]. ACLF and CLF are common, serious conditions among chronic HBV-infected patients. ACLF is defined as acute deterioration in liver function in a patient with pre-existing chronic liver disease, while CLF is the clinical decompensation of end-stage liver disease without a precipitating event. The poor survival rate of these patients is in part related to the diagnosis of liver failure at advanced stages when effective therapies are lacking. Prognosis is clearly related to the stage at which the disease is detected, and early diagnosis has resulted in a significant reduction in mortality [4,6–9]. Unfortunately, no satisfactory biomarkers are available for early diagnosis of liver failure. A differential diagnosis from non-liver failure patients is sometimes very difficult to make because biomarkers used in clinical diagnosis lack sensitivity and reliability [7–9]. For example, serum coagulopathy and bilirubin levels have been the serum marker widely used for diagnosis of liver failure; however, the cut-off levels of bilirubin vary between 5 and 20 mg/dL. Some liver failure patients, particularly during the early stages, have lower

Abbreviations: ACLF, acute-on-chronic liver failure; ALB, albumin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CHB, chronic hepatitis B subjects without cirrhosis and liver failure; CLF, chronic liver failure; HBV, hepatitis B virus; HS, healthy subjects; LC, compensated liver cirrhosis; TBil, total bilirubin.

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serum bilirubin levels. In contrast, elevated serum bilirubin and decreased prothombin activity may be seen in patients with obstruction jaundice [7–9]. Thus, it is very important to discover and validate new biomarkers for liver failure.

Recently, the search for potential biomarkers has been increasingly successful due to the application of new proteomics techniques. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry is a sensitive method that can precisely separate target proteins according to their mass-dependent velocities [10,11]. In the present study, we used MALDI-TOF mass spectrometry with ClinProt magnetic beads [weak cation exchange (WCX)] to analyze the serum peptide profiles from patients with different stages of HBV-associated chronic liver disease ranging from chronic hepatitis to liver failure. The objective of the study was to determine whether serum peptide profiling could distinguish between patients with different stages of liver disease, particularly between those chronically infected with HBV with and without liver failure.

METHODS

Clinical data and serum collection

All patients and healthy subjects were enrolled from the Tianjin Third Central Hospital, Tianjin Medical University (Tianjin, China) between October 2007 and April 2008. The study protocol was approved by the ethics committee of the Tianjin Third Central Hospital. Written informed consent was obtained from each subject.

A total of 164 samples was obtained from five groups of consecutively recruited subjects, including healthy volunteer subjects as normal controls [healthy subjects (HS); $n = 20$], and chronic Hepatitis B subjects without cirrhosis and liver failure (CHB; $n = 33$), compensated liver cirrhosis with Child–Pugh Score <7 [compensated liver cirrhosis (LC); $n = 35$], acute-on-chronic liver failure (ACLF; $n = 38$) or chronic liver failure (CLF; $n = 38$). These serum samples were divided into two groups: (i) 14 HS, 25 CHB, 26 LC, 25 ACLF and 26 CLF subjects for the training model; and (ii) 6 HS, 8 CHB, 9 LC, 13 ACLF and 12 CLF subjects for the blinded test. ACLF and CLF were diagnosed, manifesting as severe jaundice and severe coagulopathy [5,7,8] (the clinical characteristics are shown in Table 1). All fasting serum samples were collected in the morning when the patients were admitted to the hospital. The blood samples were centrifuged at 2600 *g* for 10 min at 4 °C, and the sera were stored at −80 °C until analysis.

Peptide fractionation spectra analysis using ClinProt magnetic beads

Peptide fractionation of the samples was performed with ClinProt purification reagent sets from Bruker Daltonik (Bremen, Germany). Magnetic particles with WCX beads (Bruker Daltonik) were used according to the manufacturer's instructions. Briefly, 5 μL serum was mixed with 10 μL binding solution in a thin-wall microcentrifuge tube. WCX beads (5 μL) were added and mixed thoroughly by pipetting up and down five times. The microcentrifuge tube was then placed in a magnetic bead separator in which the beads were washed three times, and the bound peptides were eluted with 5 μL elution buffer (1:1 by volume). We prepared targets by spotting 1 μL of a mixture containing 10 μL of 0.3 g/L α-cyano-4-hydroxycinnamic acid in ethanol–acetone (2:1 by volume) and 1 μL of the eluted proteome fraction on the AnchorChip™ target (Bruker Daltonik). For the proteome analysis, we used a MALDI-TOF mass spectrometer (AutoflexII; Bruker Daltonik) with the following settings: ion source 1, 20 kV; ion source 2, 18.60 kV; lens, 7.60 kV; pulsed ion extraction, 320 ns; and nitrogen pressure, 2000 mbar.

Mass calibration was performed with Sigma serum sample including 11 standard peptides at a mass range of 800 to 10 000 Da according to the ClinProt standard calibration protocol. The coefficient of variance (CV) of typical peak

Table 1 Clinical characteristics of subjects with liver disease and healthy subjects

Characteristic	Group 1 (HS)	Group 2 (CHB)	Group 3 (LC)	Group 4 (ACLF)	Group 5 (CLF)
Sex (male/female)	10/10	24/9	19/16	32/6	29/9
Age (years)	27 ± 7.37	30.06 ± 11.64	44.27 ± 10.15	54.28 ± 10.79	52.41 ± 10.92
ALT (U/L)	27.90 ± 4.73	49.79 ± 66.69	44.15 ± 20.47	617.36 ± 668.4[abcd]	51.34 ± 63.71[d]
AST (U/L)	22.00 ± 3.38	37.71 ± 51.65	45.11 ± 17.05	903.14 ± 1597.21[abcd]	66.63 ± 52.39[d]
TBil (μmol/L)	16.70 ± 3.81	17.19 ± 6.06	32.59 ± 23.55	395.05 ± 213.70[abcd]	125.52 ± 79.13[abcd]
ALB (g/L)	41.70 ± 3.33	39.14 ± 7.26	34.37 ± 3.61[ab]	29.22 ± 3.59[abcd]	24.35 ± 4.57[abcd]

ACLF, acute-on-chronic liver failure; ALB, albumin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CHB, chronic hepatitis B subjects without cirrhosis and liver failure; CLF, chronic liver failure; HS, healthy subjects; LC, compensated liver cirrhosis; TBil, total bilirubin.
a, *vs* Group 1, $P < 0.05$; b, *vs* Group 2, $P < 0.05$; c, *vs* Group 3, $P < 0.05$; d, Group 4 *vs* Group 5, $P < 0.05$.
Data are presented as the mean ± SD.

areas and intensities was between 9% and 21%, verifying the reproducibility of profile spectra for analysis. For each MALDI spot, 400 spectra were acquired during the analysis.

Statistical analysis

FlexAnalysis 3.0 and ClinProTools 2.1 (Bruker Daltonik) were used to analyze the resulting spectra. A *P*-value of <0.05 was considered statistically significant. Peptide profiling of sera from patients using the WCX beads allowed building of the classification diagnostic models based on the Genetic Algorithm (GA) and the Quick Classifier Algorithm (QC) [12,13]. Diagnostic accuracy was further valuated by a blinded test [14].

RESULTS

Serum peptide analysis across five groups

There were significantly different peptide patterns among the five test groups (HS, CHB, LC, ACLF and CLF) (Fig. 1). A total of 85 differentially expressed peptide peaks was detected across the five groups. We analyzed these data with the GA method, and a classification diagnostic model was obtained with characteristic peptide peaks at 4053 m/z, 3506 m/z, 4963 m/z, 9289 m/z, 2628 m/z, 3193 m/z and 6432 m/z. The model had a diagnostic capability of 88.76% overall, while it was 100% for HS, 92% for CHB, 80% for LC, 83.33% for ACLF and 88.46% for CLF. The predictive capability of the model was 54.27%.

In comparing the average statistical peak areas among the five groups, we also found that the peak at 6432 m/z was significantly downregulated and correlated with the severity of liver disease ($P < 0.000001$). The peptide at 9289 m/z showed significant upregulation in CHB and LC patients, while the peptide with a mass at 4963 m/z showed significant upregulation in ACLF and CLF patients compared with other groups ($P < 0.000001$). These peptides were chosen by bioinformatics analysis as the most significant different peaks. They were also chosen as characteristic peptides in the classification diagnostic models for the five-group comparisons.

Serum peptide pattern comparisons between two groups

The serum peptide patterns from CHB, LC, ACLF, CLF and HS groups were analyzed and then two-way comparisons were performed. Classification diagnostic models of each liver disease state generated on GA or QC classifiers are summarized in Tables 2–5. There were remarkable pattern differences of mass spectra among samples from healthy subjects and patients with different liver disease. The predictive capability and accuracy of the classification models for ACLF or CLF *vs* CHB were remarkably higher than those *vs* LC. The predictive capability of the classification model for ACLF or CLF *vs* CHB was 91.41% and 97.99%, respectively, while that for *vs* LC was 87.72% and 82.18%, respectively. The classification model of ACLF *vs* CLF had a relatively lower prediction capability (75.05%). These classification models were validated by additional subjects in a blinded test that generated recognition accuracy with a range from 67–100%.

DISCUSSION

Currently available serum markers for the detection of liver failure are limited in sensitivity and specificity. In clinical practice, the international normalized ratio (INR) or prothrombin activity (PTA) and bilirubin are the most commonly used serological biomarkers for the detection of liver failure. Their diagnostic efficacies are known to be very low [8,9].

Serum samples are readily available in medical practice, and they contain complex peptides that correlate with

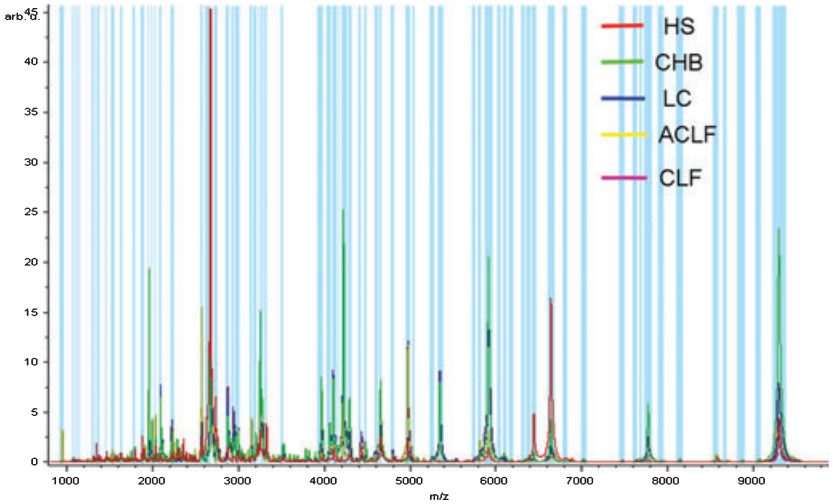

Fig. 1 Serum peptide profiles from healthy subjects and subjects with different liver diseases. ACLF, acute-on-chronic liver failure; CHB, chronic hepatitis B subjects without cirrhosis and liver failure; CLF, chronic liver failure; HS, healthy subjects; LC, compensated liver cirrhosis.

Table 2 Classification diagnostic models for CHB, LC, ACLF or CLF *vs* HS

Group	m/z	Diagnostic capability (%) (algorithm)	Predictive capability (%)	Accuracy by blinded test (%)
CHB *vs* HS	6630, 1329, 2721, 1886, 3240	100 (overall, GA) 100 (CHB) 100 (HS)	100	100 (8/8, CHB) 100 (6/6, HS)
LC *vs* HS	2658, 4208, 6630	100 (overall, QC) 100 (CHB) 100 (HS)	96.67	78 (7/9, LC) 100 (6/6, HS)
ACLF *vs* HS	6432, 1329, 2680	100 (overall, GA) 100 (CHB) 100 (HS)	98.98	100 (13/13, ACLF) 100 (6/6, HS)
CLF *vs* HS	2658, 4963, 5905, 6630	98.08 (overall, QC) 96.15 (CLF) 100 (HS)	94.67	92 (11/12, CLF) 100 (6/6, HS)

ACLF, acute-on-chronic liver failure; CHB, chronic hepatitis B subjects without cirrhosis and liver failure; CLF, chronic liver failure; GA, genetic algorithm, HS, healthy subjects; LC, compensated liver cirrhosis; QC, quick classifier algorithm.

Table 3 Classification diagnostic models for LC, ACLF or CLF *vs* CHB

Group	m/z	Diagnostic capability (%)	Predictive capability (%)	Accuracy by blinded test (%)
LC *vs* CHB	6088, 6663, 9289, 2931, 4233	98 (overall, GA) 100 (LC) 96 (CHB)	79.8	67 (6/9, LC) 100 (8/8, CHB)
ACLF *vs* CHB	9289, 2862, 6088, 6630, 3258	100 (overall, GA) 100 (ACLF) 100 (CHB)	91.41	92.3 (12/13, ACLF) 100 (8/8, CHB)
CLF *vs* CHB	9289, 6375, 3260, 2941, 4209	100% (overall, GA) 100 (CLF) 100 (CHB)	97.99	92 (11/12) 100 (8/8, CHB)

ACLF, acute-on-chronic liver failure; CHB, chronic hepatitis B subjects without cirrhosis and liver failure; CLF, chronic liver failure; LC, compensated liver cirrhosis; GA, genetic algorithm.

Table 4 Classification diagnostic models for ACLF or CLF *vs* LC

Group	m/z	Diagnosic capability (%)	Predictive capability (%)	Accuracy by blinded test (%)
ACLF *vs* LC	3141, 6630, 6589, 7765, 2928, 3954, 9289, 6665 2080, 1978, 2989, 5805, 4963, 6088, 5903, 4920 1944, 2560, 6432, 2228, 6374, 4419, 4433, 1864	97.92 (overall, GA) 95.87 (ACLF) 100 (LC)	87.72	85 (11/13, ACLF) 100 (9/9, LC)
CLF *vs* LC	2941, 3193, 5247, 4643, 6432, 4614, 4116	98.08 (overall, GA) 96.15 (CLF) 96 (LC)	82.18	83 (10/12, CLF) 100 (9/9, LC)

ACLF, acute-on-chronic liver failure; CLF, chronic liver failure; LC, compensated liver cirrhosis; GA, genetic algorithm.

Table 5 Classification diagnostic models for ACLF *vs* CLF

Group	m/z	Diagnostic capability (%)	Predictive capability (%)	Accuracy by blinded test (%)
ACLF *vs* CLF	9289, 2925, 4679, 1518, 2899	88.15 (overall, GA) 84 (ACLF) 92.31 (CLF)	75.05	77 (10/13,ACLF) 75 (9/12, CLF)

ACLF, acute-on-chronic liver failure; CLF, chronic liver failure; GA, genetic algorithm.

biological events. Comparison of proteomic or peptide patterns can reveal significant pathophysiological changes quantitatively; therefore, such studies are popular for the discovery of biomarkers of various diseases. Comprehensive proteomic techniques have been used to research cancer and other diseases [13–16]. It is well known that the liver plays an important central role in metabolism, and liver failure induces severe metabolic disturbance [17–19]. Serum proteomic or peptide analysis would be useful for identifying potential biomarkers for liver disease. Proteomic analysis of serum derived from subjects with liver disease is an emerging technique for the identification of biomarkers indicative of disease severity and progression [20–22]. The use of ClinProt magnetic beads is a new proteomics approach for the discovery of potential diagnostic biomarkers. This method uses different chemical chromatographic surfaces on an outer layer of magnetic beads to selectively purify certain subsets of protein. Proteins bound to the magnetic beads are then analyzed by MALDI-TOF mass spectrometry, and the discriminatory proteins or peptides can be identified by downstream bioinformatics analysis [10,23,24].

The distribution of proteomic data is usually too wide to fit normal distribution well. Conventional approaches with mean values and SD often lead to an inaccurate interpretation of available data. Therefore, it is necessary to evaluate multiple algorithms for biomarker analysis to improve diagnostic efficacy [12,13,23]. In our study, FlexAnalysis 3.0 and ClinProTools 2.1 were used to analyze the resulting spectra, and the classification diagnostic model of liver disease was obtained based on two algorithms: GA and QC. Sensitivity and specificity were further validated by a blinded test. The classification model based on analysis of the five test groups had a diagnostic capability of 88.76% and a prediction capability of only 54.27% overall. Indeed, the GA and QC algorithms used in our study were not effective for intraspectral analysis, because their diagnostic efficacy was limited when the five groups were analyzed simultaneously. Therefore, we focused on two-way comparisons between the ACLF, CLF, LC, CHB and HS groups.

Significant differences exist in the prognoses for ACLF, CLF, CHB and LC patients, and it is important to differentiate between those with and without liver failure. Some proteomics and metabonomic studies of liver failure have been reported recently, but these reports focussed on animal models and were limited in comparisons with healthy subjects [25–28]. In our study, we investigated different liver disease stages in humans. Multiple or two-way comparisons of different liver disease conditions were performed. In this work, we demonstrated that there are significantly different profiles of serum peptides between healthy subjects and patients with various states of liver disease. We also found the predictive capability and accuracy of the classification models for ACLF or CLF *vs* CHB were remarkably higher than those *vs* LC. The predictive capability of the classification model for ACLF or CLF *vs* CHB was 91.41% and 97.99%, respectively, while that for ACLF or CLF *vs* LC was 87.72% and 82.18%, respectively. The classification model of ACLF *vs* CLF had a relatively lower prediction capability (75.05%). As the difference in severity of liver disease decreased, the predictive capability of the classification model gradually decreased. These findings are consistent with pathophysiological changes during the progression of liver failure [17,19].

In addition, we found some statistically significant upregulation or downregulation of peak intensities related to the severity of liver disease. These peptides maybe useful for differentiating the different stages of liver injury. From the comparison of average peak areas across the five groups, we found some characteristic peptides upregulated or downregulated in ACLF and CLF. Schwegler *et al.* [20] also reported that the mean intensity of the 5808 m/z peak increased with disease severity during the progression of chronic hepatitis C to cirrhosis and hepatocellular caricinoma. The results indicate that these serum peptides, as followed by proteomic analysis, are potential new biomarkers to distinguish severe liver disease. Further validation of these markers is needed.

In conclusion, in our study we demonstrated that serum peptide profiling can provide discrimination of peptide peaks to classify different stages of chronic liver diseases. MALDI-TOF mass spectrometry with ClinProt magnetic beads is an effective tool to investigate potential diagnostic biomarkers for liver disease, particularly for liver failure. Our research will be valuable for the detection of liver failure and for future investigations into the mechanisms of liver failure.

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