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Serum peptidome patterns of hepatocellular carcinoma based on magnetic bead separation and mass spectrometry analysis

Ke Li, Wei-Hua He, Nai-Jun Fan, Chun-Fang Gao, Guang Zhao, Gao-Bin Sun, Guo-Bao Zheng
Institute of Anal-colorectal Surgery, No. 150 Central Hospital of PLA, Luoyang, China

ABSTRACT

Background/Aims: The only hope for a cure from hepatocellular carcinoma (HCC) rests on early diagnosis. The present study aims to determine serum peptidome patterns for early diagnosis of HCC.

Materials and Methods: To identify novel peptidome patterns for diagnosing HCC, serum from31 healthy volunteers and 32 HCC patients were subjected to a comparative proteomic analysis using a ClinProt Kit combined with mass spectrometry (MS). This approach allows the determination of peptidome patterns that are able to differentiate the HCC from healthy volunteers. For further validation, the diagnostic and differential diagnostic capabilities of the peptidome patterns were verified blindly by an independent group of sera consisted of 31 HCC, 23 liver fibrosis and 33 healthy volunteers.

Results: A Quick Classifier Algorithm was used to construct the peptidome patterns for the identification of HCC from the control samples. One of the identified peaks at m/z 7771 was used to construct the peptidome patterns with almost 100% accuracy. Furthermore, the peptidome patterns could also differentiate the validation group with high accuracy.

Conclusion: These results suggest that the ClinProt Kit combined with MS achieves significantly high accuracy for HCC diagnosis and differential diagnosis.

Keywords: Hepatocellular carcinoma, proteomics, diagnosis, mass spectrometry

Original Article

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most commonly diagnosed cancer in worldwide (1). Despite advances in surgical therapy for HCC, the overall prognosis of patients has not improved markedly during the past few decades due to the fact that most patients have locally-advanced or disseminated diseases at diagnosis. Currently, no satisfactory biomarkers are available to HCC screening. Early diagnosis of HCC is therefore critical for guiding the therapeutic management and improving prognosis.

Proteomics, which concerns comprehensive protein profile changes caused by multiple gene alterations, has emerged as a valuable tool in scientific medicine (2-6). Human serum contains thousands of proteolytically derived peptides called peptidomes, which may provide a robust correlation with the physiologic and pathologic processes in the entire body (7,8). The panels of peptidome markers might be more sensitive and specific than conventional biomarker approaches (9). Proteomic pattern by mass spectrometry is one of the most promising new approaches for early identification of disease from health volunteers. Advances in mass spectrometry (MS) now permit the display of hundreds of small- to medium-sized peptides using only microliters of serum (10,11). Preliminary studies have shown that great interest has been focused on the low-molecular-weight region, particularly on peptides smaller than 20 kDa, which may provide a novel means of diagnosing cancer and other diseases (8,12,13).

Matrix-assisted laser desorption/ionization time-of-flight MS (MALDI-TOF MS) can detect peptides with low molecular weights with the necessary sensitivity and resolution, which makes it a useful technique for serum peptide profiling. Furthermore, for accurate MS analyses, the peptidome fractionation procedure and

Address for Correspondence: Gao Chun-Fang, Institute of Anal-colorectal Surgery, No. 150 Central Hospital of PLA, Luoyang, China
E-mail: chunfanggao@126.com
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the preanalytical conditions of peptidome mapping must be carefully assessed (14). Magnetic beads (MBs), based on nanomaterials, have been developed and considered as a promising material for convenient and efficient enrichment of peptides and proteins in biological samples (15,16). The combination of MALDI-TOF MS enables the high throughput and sensitive investigation of peptides and proteins.

In the current study, a well-defined novel technology platform called ClinProt (Bruker Daltonics, Ettlingen, Germany) was used for early screening of HCC (17,19). A diagnostic model, which consists of one differentially expressed peptides, was established and validated by the QC Algorithm, by which different groups were effectively discriminated. Then, the diagnostic model was further verified using blinded samples from HCC and healthy volunteers. Thus, the preliminary work for the early and differential diagnoses of HCC was completed from an integrated perspective of peptide mass patterns.

MATERIALS AND METHODS

Reagents and instruments

The AutoFlex III MALDI-TOF mass spectrometer, MTP 384 target plate polished steel, α-cyano-hydroxycinnamic acid (CHCA), MB-WCX kit, and peptide calibration standard were purchased from Bruker Daltonics (Leipzig, Germany). The trifluoroacetic acid (TFA) and acetonitrile (ACN) were purchased from Alfa Aesar (Ward Hill, MA, USA), Sigma (St. Louis, MO, USA), and Roche Diagnostics GmbH (Sandhofer Strasse, Germany), respectively.

Patients and sample collection

With their consent, 64 healthy subjects (blood donor volunteers), 23 liver fibrosis patients and 63 HCC patients were enrolled into the study, and the blood samples were collected. Endoscope was performed on all healthy subjects to exclude the presence of incidental digestive tract masses. The HCC patients underwent clinical staging, surgical lesion excision, and follow-up sessions.

Serum samples were prepared by collecting blood in a vacuum tube and allowing it to clot for 30 min at room temperature. About 1 mL of serum was obtained after centrifugation at 2000 rpm for 10 min and stored in small aliquots at -80 °C until analysis.

Study design

The data set, including 64 control and 63 HCC patients, was randomly split into two groups. The 23 liver fibrosis patients were recruited in the second group. The clinical characteristics of the HCC patients are shown in Table 1. The first group (model construction data set: 31 healthy volunteers and 32 HCC patients) was used for the identification of signals related to peptides expressed differentially among HCC patients compared with control subjects. The group was also used for the pattern recognition. The second group (external evaluation data set: 33 healthy volunteers, 23 liver fibrosis patients and 31 HCC patients) was used for the blind independent pattern validation of the cluster.

The gender ratios (male/female) of the healthy volunteers and HCC patients were 1.46 and 2.44, respectively. The mean ages (years) of the healthy volunteers and HCC patients were 54.63±1.37 and 58.48±10.60, respectively. The age and gender ratio differences between the healthy volunteers in the model construction group and those in the external evaluation data set were not significant. No significant differences were also observed for the ages and gender ratios of the HCC patients and healthy volunteers, as well as for the TNM stages of the HCC patients in the model construction and external evaluation groups.

Sample purification

WCX-MBs were used for the peptidome separation of samples following the manufacturer's standard protocol (20). First, 10 µL of WCX-MB binding solution and 10 µL of WCX beads were combined in a 0.5 mL microfuge tube after thoroughly vortexing both reagents. Second, 5 µL of serum sample was added and mixed by pipetting up and down. Third, the microfuge tubes were then placed in an MB separator (MBS) and agitated 10 times. The beads were collected from the tube walls 1 min later. Fourth, the supernate was carefully removed using a pipette. Fifth, 100 µL of WCX-MB wash buffer was added into tubes, which were again agitated 10 times in the MBS. The beads were then collected from the tube walls, and the supernate was carefully removed using a pipette. After three washes, 5 µL of the WCX-MB elution buffer was added to disperse the beads in tubes by pipetting up and down. The beads were collected from the tube walls after 2 min, and the clear supernate was transferred into fresh tubes. Then, 5 µL of WCX-MB stabilization solutions were added to the collected supernate and mixed intensively using a pipette. The mixture was then ready for spotting onto MALDI-TOF MS targets and measurement. Finally, prior to the MALDI-TOF MS analysis, the targets were prepared by spotting 1 µL of the proteome fraction on the polished steel target (Bruker Daltonics, Bremen, Germany). After

Table 1. Clinical characteristics of hepatocellular carcinoma (HCC) patients recruited in model construction group and external validation group

Clinical characteristics	Model construction group (n=60)	Evaluation group (n=59)	p value
Gender: male/female	34/26	35/24	0.937
Age(years, $\bar{X}$±SD)	58.24±8.21	57.46 9.39	0.912
TNM stage			0.381
I	18	16	
II	42	43	

§Pearson Chi-Square test.
#Independent-Sample T Test.

air-drying, 1 µL of 3 mg/mL CHCA in 50% ACN and 50% Milli-Q with 2% TFA was applied onto each spot, and then, the target was air-dried again (cocrystallization). The peptide calibration standard (1 pmol/µL peptide mixture) was applied for machine calibration.

MS analysis

For proteome analysis, a linear Autoflex III MALDI-TOF mass spectrometer was used with the following settings: ion source 1, 20.00 kV; ion source 2, 18.60 kV; lens, 6.60 kV; and pulsed ion extraction, 120ns. Ionization was achieved via irradiation with a crystal laser operating at 200 Hz. For the matrix suppression, a high gating factor with signal suppression up to 600 Da was used. The mass spectra were recorded in linear positive mode. Mass calibration was performed using the calibration mixture of the peptides and proteins in the mass range of 1–12 kDa. Three MALDI preparations (MALDI spots) were measured for each MB fraction. For each MALDI spot, 1600 spectra were quantified (200 laser shots at eight different spot positions). The spectra were recorded automatically using the Autoflex Analysis software (Bruker Daltonics, Bremen, Germany) for the fuzzy-controlled adjustment of the critical instrument settings to generate raw data with optimized quality.

Bioinformatics and statistical analysis

The ClinProt Tools software 2.2 (Bruker Daltonics) was used to analyze all serum sample data derived from either the patients or the normal controls. The data analysis began with raw-data pretreatment, including baseline subtraction of spectra, normalization of a set of spectra, internal peak alignment using prominent peaks, and a peak-picking procedure. The pretreated data were then used for visualization and statistical analysis in ClinProt Tools. Statistically significant differences in peptide quantity were determined using Welch's *t*-tests. The significance was set at $p < 0.05$. The class prediction model was set up using the QC Algorithm. Then, the classified peptidome patterns were constructed. To determine the accuracy of the class prediction, a cross-validation was first implemented. Twenty percent of the samples from the model construction group were randomly selected as a test set and the remaining samples were taken as a training set in the class predictor algorithm. Second, by designing a double-blind test, the samples of external evaluation group were classified using the classified peptidome patterns constructed by the QC Algorithm.

Statistical methods and evaluation of assay precision

Each spectrum recorded using the MALDI-TOF MS was analyzed with Autoflex Analysis to detect the peak intensities of interest and with ClinProt™ software (Bruker Daltonics) to compile the peaks across the spectra recorded from all samples. This setup allowed differentiation between the cancer and the control samples. To evaluate the precision of the assay, the within- and between-run variations were determined using multiple analyses of bead fractionation and MS for two plasma samples. For the within- and between-run variations, three peaks with various intensities were examined. The within-run imprecision was determined by evaluating the coefficient variations (CVs) for each sample, using eight assays within a run, and then the between-run imprecision was determined by performing eight different assays over a period of seven days. SPSS 16.0 was used to analyze the clinical characteristics of the volunteers using a χ^2 test or a *t*-test. The significance was set at $p < 0.05$.

RESULTS

For the reproducibility of the protein profiling, the within- and between-run reproducibility of two samples was determined via WCX-MB fractionation and MALDI-TOF MS analysis. In each profile, three peaks with different molecular masses were selected to evaluate assay precision. Despite varying peptide masses and spectral intensities, the peak CVs were all <4% and <10% in the within- and between-run assays, respectively. These values were consistent with the reproducibility data for the Protein Biology System reported by Bruker Daltonics.

In the pilot study, the differences between the serum proteome profiles of HCC patients and healthy subjects were evaluated. The mass spectra from 1 kDa to 20 kDa were obtained using MALDI-TOF MS in linear mode. The representative mass spectra of the prefractionated sera of the model construction group are reported in Figure 1. On average, about 169 signals common to the two groups have been detected in this mass range and about 16 were identified by the ClinProt software with a statistically different area ($p < 0.05$ using the *t*-test) in the model construction population, including 1 upregulated and 15 downregulated peptides, respectively (Table 2).

Classification models were developed to classify between the HCC and healthy samples. The use of individual peaks as diagnostic biomarkers for HCC was addressed using QC algorithm analysis. First, the HCC patients and healthy volunteers were compared. Second, all detected peaks were analyzed using ClinProt 2.2 to generate the cross-validated classification models. The optimized model resulted in the following correct sample classification. One peptide ion signatures (m/z 7771) were provided as a class prediction for a cross-validation set to

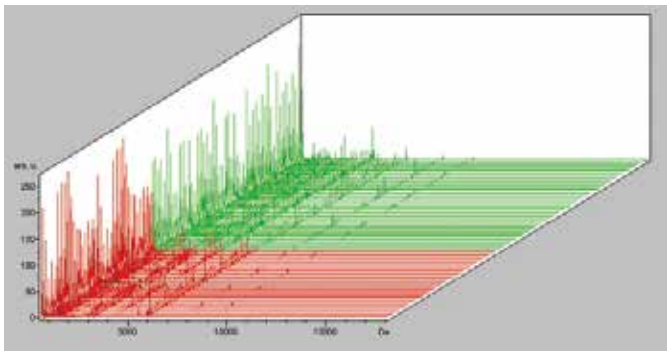

Figure 1. View of the aligned mass spectra of the serum protein profiles of the model construction group (red represents healthy subjects, and blue denotes HCC patients) obtained using MALDI-TOF after purification with WCX-MBs.

Table 2. Statistics of the 16 dysregulated proteins/peptides in hepatocellular carcinoma (HCC) patients compared with health individuals

Mass	HCC▲ (Average±SD)	Healthy volunteers▲ (Average±SD)	Regulation in HCC	p value□
7643.01	0.25±0.12	0.92±0.58	↓	0.000202
7927.69	0.34±0.27	1.09±0.61	↓	0.000202
7833.44	3.72±3.73	16.79±11.58	↓	0.000289
4397.32	2.96±1.94	9.68±6.18	↓	0.0004
7771.17□	29.82±13.68	111.35±17.93	↓	0.000494
5027.39	2.58±1.35	7.50±4.86	↓	0.000636
7896.09	0.85±0.81	3.31±2.48	↓	0.000773
8147.79	0.62±0.54	1.86±1.19	↓	0.000773
9359.98	3.28±4.60	11.72±8.40	↓	0.00146
4965.18	7.25±6.49	29.35±24.79	↓	0.00165
6636.05	0.34±0.18	1.12±0.45	↓	0.00165
1082.55	16.22±3.70	10.13±6.01	↑	0.00165
4272.16	50.13±14.56	101.95±38.12	↓	0.00165
9524.77	0.05±0.04	0.12±0.08	↓	0.00177
2510.81	8.59±5.01	27.28±12.89	↓	0.00326
2354.46	6.69±3.53	18.35±4.61	↓	0.00416

*The peptide selected for model construction.
▲Peak area.
★p value calculated with the t-test; value lower than 0.05 suggest statistical relevance.

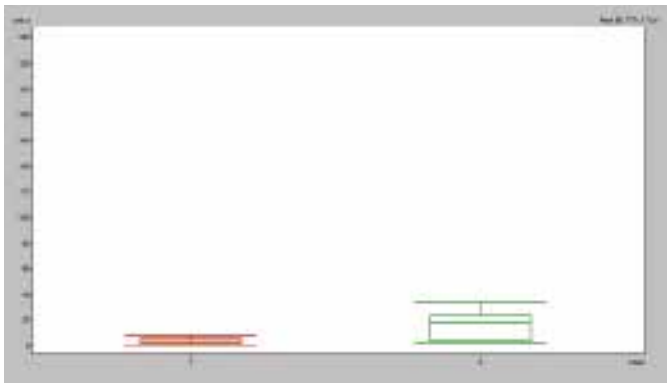

Figure 2. Box-and-whisker plot calculated from the areas of the peak (m/z 7771) used in the cluster for the two studied populations (p<0.01). Group 1 represents HCC patients, and Group 2 represents healthy volunteers.

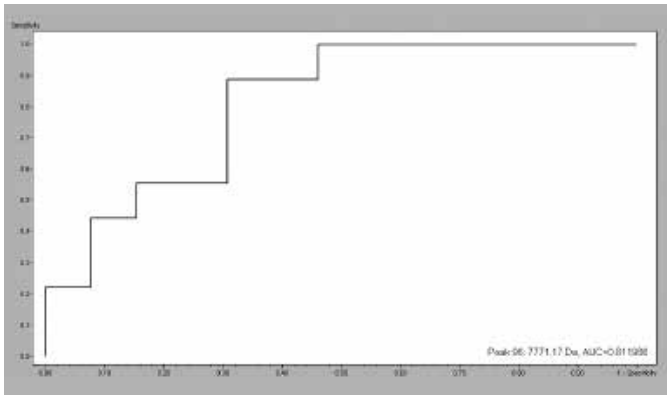

Figure 3. The receiver operating characteristic curves of the peak (m/z 7771) selected for the diagnostic model. AUC: Area under the receiver operating characteristic curve.

discriminate the HCC patients from healthy volunteers, which achieved 100% recognition and 90.42% cross-validation accuracy. The areas of the peak (m/z 7771) in the spectra of HCC patients were statistically different from those of the healthy volunteers (Figure 2).

The preliminary statistical analysis was performed for each single marker and signal cluster using the receiver operating characteristic curve analysis. The area under curve (AUCs) of peak at m/z 7771 was 0.81, which shows a highly accurate test, according to the criteria suggested by Swets [21] (Figure 3).

To verify the accuracy of the established QC classification model with the adopted peptides, another group of samples was introduced (not used in the model construction), which consisted of 31 HCC and 33 healthy subjects. As a result, the model correctly classified 96.77% (30/31) of the HCC (sensitivity), 82.61% (19/23) of liver fibrosis (specificity) samples and 100% (33/33) of the healthy (specificity) samples.

DISCUSSION

The usefulness of multiple markers for diagnosis, prognosis, and prediction of the risk of developing diseases or their complications is now widely recognized (7,22). Various proteomic approaches have been applied to biomarker discovery using biological fluids. Interestingly, low-molecular-weight peptides, such as S100A8 and fibrinogen, have been recognized to play important roles in physiologic and pathologic processes and could be used as relevant biomarker candidates (23,24). Recently, the mass spectrum that directly detects and differentiates short peptides has offered a promising approach for peptidomic biomarker discovery (8,12,25-27).

Compared with genomic approaches, proteomic analysis has the advantage of visualizing co- and posttranslational protein modifications, which are possibly relevant to biologic functions. Alternative approaches for measuring polypeptides, such as the surface-enhanced laser desorption and ionization, recently reported by several groups, have several disadvantages, such as low resolution and the loss of most proteins and peptides (28-30). MALDI is a soft ionization technique used in MS that allows the analysis of biomolecules such as proteins, peptide sugars, and large organic molecules. As a powerful tool for surveying the complex patterns of biologically informative molecules, MALDI-TOF MS protein/peptide profiling has been applied in proteomics biomarker research and has become a promising tool in cancer biomarker research (25,31,32).

In the present study, by integrating short peptide purification with WCX-MBs, peak intensity detection with MALDI-TOF MS, and profile analysis with ClinProt Tools software 2.2, a series of differentially expressed short peptides in the sera of HCC patients has been successfully detected. A comparative case control analysis between HCC and healthy subjects was performed. Peptidomic maps associated with the disease were drawn. The results show that compared with the normal controls, the HCC patients share 16 significantly differentiated peptides, including 1 upregulated and 15 downregulated peptides. The current knowledge on cellular regulation indicates that many networks operate at the epigenetic, transcriptional, and translational levels. Genomic and proteomic technologies will further help us understand the intracellular signaling and gene transcription systems, as well as the protein pathways that connect the extracellular microenvironment to the serum or plasma macroenvironment of cancer (33). These 16 interesting significantly differentiated peptides may provide further evidence for understanding the occurrence and progress of HCC. In particular, the prominent peptides that have a greater than twofold change in intensity, such as m/z 7833, 4397 and 9359 may be defined as the leading differential peptides associated with HCC, which are worthy of further sequence determination and functional analysis.

Using QC algorithm analysis, classification models were developed to classify samples between normal controls and HCC. The peptide at m/z 7771 achieved a recognition capacity and a cross-validation of close to 100% (83.87% specificity and 93.75% sensitivity) to discriminate HCC from normal volunteers. The blinded verification of the QC classification model proved the correct classification of 96.77% (30/31) of the HCC patients, 82.61% (19/23) of liver fibrosis patients and 100% (33/33) of the healthy volunteers. This result demonstrates that the QC Algorithm is effective in facilitating the construction of a sensitive and specific diagnostic and different diagnositic model.
This study is the focused on screening for HCC-related short peptides in sera by combining WCX-MBs and MALDI-TOF MS. The classification model that we have set up has applications in providing alternatives for HCC diagnosis or differential diagnosis and may provide a better understanding of HCC pathogenesis or help in tailoring the use of chemotherapy for each patient, finally resulting in improved patient outcomes.

In conclusion, peptidome patterns from WCX-MB-purified serum samples were directly profiled with MALDI-TOF MS and a peptidome model that differentiated HCC from the control samples was constructed with high sensitivity and specificity. Despite the high sensitivity and specificity, the number of specimens analyzed in this study was relatively small, which may limit the validity of the results. The next step in our study will be to analyze larger patient cohorts and to run blinded samples to confirm the usefulness of the currently identified peptides for HCC diagnosis. After this confirmation, the biomarkers of the interest will then be isolated and identified and their biological role in HCC pathogenesis will be studied.

Conflict of Interest: No conflict of interest was declared by the authors.

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