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Diagnosis of Gallbladder Cancer Using Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Profiling

Yonghua Mou, MD, Renwei Xing, MD and Chibo Liu, MD

Abstract: Proteomic fingerprint technology combining magnetic beads with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry was used to profile and compare the serum proteins from 45 patients with gallbladder cancer and 50 healthy blood donors. The proteomic patterns were identified; the tree model of biomarkers was constructed and evaluated using the Biomarker Patterns Software. The model tree was constructed based on the 3 biomarkers (5913 Da, 6181 Da and 13,752 Da), which generated excellent separation between the gallbladder cancer and control groups. The sensitivity was 86.7% and the specificity was 93.3%. The blind test data showed a sensitivity of 80% and a specificity of 90%. Taken together, our studies suggested that biomarkers for gallbladder cancer could be discovered in serum by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry combined with the use of magnetic beads. The pattern of combined markers would provide a powerful and reliable diagnostic method for gallbladder cancer with high sensitivity and specificity.

Key Indexing Terms: MALDI; Gallbladder cancer; Biomarker; Proteomics; Magnetic beads. **[Am J Med Sci 2012;343(2):119–123.]**

Gallbladder cancer is one of the most common cancers and the fifth leading death in the digestive tract tumors in the world. Genetic abnormality plays critical roles in the development and progression of gallbladder cancer cells. Besides the chemotherapy, currently there lack efficient approaches in clinical practices to prevent gallbladder cancer. This suggests an urgent need in technology to predict and diagnose the patients in early stages of gallbladder cancer with high sensitivity and specificity. The proteomics techniques showed significant contributions to the cancer diagnosis and the finding of new antitumor drugs in recent years.[1] The proteomic patterns in given body fluid sample could be used for the cancer early detection and measuring the therapeutic efficacy of anticancer drugs.[2] For instance, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) offers high-throughput protein profiling.[3,4] When combined with appropriate bioinformatics tools,[5] the nondisease-related artifacts could be easily removed from the group of potential biomarkers.

In this study, we analyzed serum samples from patients with gallbladder cancer with MALDI-TOF MS in an attempt to identify a signature pattern for the disease diagnosis. The results showed several potentially candidate biomarkers for gallbladder cancer diagnosis. Additionally, the diagnostic model tree was established, which could differentiate patients with gallbladder cancer from healthy controls efficiently.

METHODS

Patients

Experiments were performed in Taizhou Municipal Hospital, Zhejiang, China, in August 2010. Studies were conducted on Chinese patients diagnosed with gallbladder cancer (n = 45, 21 men, 24 women; ages from 33 to 64 with an average of 45.43 years) at Taizhou Municipal Hospital and the First Affiliated Hospital of Medical College, Zhejiang University. All patients with gallbladder cancer were diagnosed according to combined clinical criteria, including type-B ultrasonic, with a combination of computed tomography, positron emission tomography or both and further confirmed by histopathological analysis. Comparative studies were also performed using controls (50 were healthy volunteers, 19 men, 31 women; ages from 27 to 72 years with an average of 49.22 years). The studies were approved by the local Ethics Committee of Taizhou Municipal Hospital and had the informed written consent of the patients and volunteers. The blood samples were collected in 5 mL BD Vacutainers without anticoagulation and allowed to clot at room temperature for up to 1 hour; the samples were then centrifuged at 4°C for 5 minutes at 10,000 rpm. The blood samples were withdrawn at the time of diagnosis for all the patients. The sera were frozen and stored at −80°C for future analysis. The patients and serum samples were then divided into 2 groups: the "training" set and the blinded "test" set (Table 1).

Weak Cation Exchange Magnetic Beads Analysis

Sample pretreatments and proteomic analysis in the proteomic profiling analysis and the serum samples from the diseased and control groups were randomized and blinded to investigators. Serum samples were pretreated with weak cation exchange (WCX) magnetic beads (SED) (Beijing SED Science & Technology); 10 μL of each serum sample was mixed with 20 μL of U9 solution (9 mol/L urea, 2% CHAPS and pH 9.0) in a 0.5 mL centrifuge tube and incubated for 30 minutes at 4°C. Denatured serum samples were diluted with 370 μL binding buffer (50 mmol/L sodium acetate, 0.1% Triton X-100 and pH 4.0). At the same time, 50 μL of WCX magnetic beads were placed in a PCR tube, and the tube was placed in a magnet separator for 1 minute, after which the supernatant was discarded carefully by using a pipette. The magnetic beads were then washed twice with 100 μL binding buffer. Then 100 μL of the diluted serum sample was added to the activated magnetic beads, mixed and incubated for 1 hour at 4°C, after which the beads were washed twice with 100 μL binding buffer.

Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry

After binding and washing, the bound proteins were eluted from the magnetic beads using 10 μL of 0.5% trifluo-

Correspondence: Chibo Liu, MD, Department of Clinical Laboratory, Taizhou Municipal Hospital, Taizhou, Zhejiang 318000, China (E-mail: liuchibo@126.com).

TABLE 1. Serum samples used in training and testing sets

Samples	Training set	Blind set	Total
Gallbladder cancer	30	15	45
Healthy volunteers	30	20	50
Total	60	35	95

roacetic acid. Then, 5 μL of the eluted sample was diluted 1:2-fold in 5 μL of SPA (saturated solution of sinapinic acid in 50% acetonitrile with 0.5% trifluoroacetic acid). Two microliters of the resulting mixture was aspirated and spotted onto the gold-coated ProteinChip array. After air drying for 5 minutes at room temperature, protein crystals on the chip were scanned with the ProteinChip (Model PBS IIc) reader (Ciphergen) to determine the masses and intensities of all peaks over the range m/z 1000 to 50,000. The reader was set up as follows: mass range (1000–50,000 Da), optimized mass range (1000–20,000 Da), laser intensity (200) and sensitivity (9). Mass calibration was performed using an all-in-one peptide reference standard which contained vasopressin (1084.2 Da), somatostatin (1637.9 Da), bovine insulin β chain (3495.9 Da), human insulin recombinant (5807.6 Da) and hirudin (7033.6 Da) (Ciphergen Biosystems, Fremont, CA). The default background subtraction was applied, and the peak intensities were normalized using the total ion current from a mass charge of 1000 to 50,000 Da. A biomarker detection software package (Ciphergen Biomarker Wizards, Ciphergen Biosystems) was used to detect protein peaks (Figure 1). Protein peaks were selected based on a first pass of signal-noise ratio of 3 and a minimum peak threshold of 20% of all spectra. This process was completed with a second pass of peak selection at 0.2% of the mass window, and the estimated peaks were added. These selected protein peaks were averaged as clusters and were exported to a commercially available software package (Biomarker Patterns, Ciphergen Biosystems) for further classification analysis.

Detection and Statistical Data Analysis

The profiling spectra of serum samples from the training set were normalized using total ion current normalization by Ciphergen's ProteinChip Software (version 3.1). Peak labeling was performed by Biomarker Wizard software 3.1 (Ciphergen Biosystems). A 2-sample t test was used to compare mean normalized intensities between the case and control groups. The P value was set at 0.01 to be statistically significant. The intensities of selected peaks were then transferred to Biomarker Pattern Software (BPS) to construct the classification tree of gallbladder cancer. Briefly, the intensities of the selected peaks were submitted to BPS as a "Root note." Based on peak intensity, a threshold was determined by BPS to classify the root node into 2 child nodes. If the peak intensity of a blind sample was lower than or equal to the threshold, this peak would be labeled as "left-side child node." Peak intensities higher than the threshold would be marked as "right-side child node." After rounds of decision making, the training set was found to be discriminatory with the least error.

All the protein peak intensities of samples in the test set were evaluated by BPS using the classification model. The gallbladder cancer and control samples were then discriminated based on their proteomic profile characteristics. The sensitivity was defined as the probability of predicting gallbladder cancer cases, and the specificity was defined as the probability of predicting control samples. A positive predictive value reflected the probability of gallbladder cancer if a test result was positive.

RESULTS

Detection of the Protein Peaks

Proteomic data from the samples of the training set (consisting of 30 patients with gallbladder cancer and 30 healthy volunteers) were analyzed with Biomarker Wizard software 3.1. Up to 209 protein peaks per spot were detected between m/z 2000 and m/z 50,000, which showed the effectiveness of the MALDI technology in resolving low molecular weight proteins (<15,000) (Figure 1).

Protein Fingerprint Analysis of Serum Samples in Patients With Gallbladder Cancer and Healthy Controls

The protein profile of the serum samples from the 30 patients with gallbladder cancer and the 30 healthy controls was extracted by magnetic beads and examined with MALDI-TOF MS. The data were analyzed by Biomarker Wizard Version 3.1; there were statistical differences of 3 protein peaks located at 5913 Da, 6181 Da and 13,752 Da in comparisons of the 2 groups (Figure 2); the intensity of protein peaks at 5913
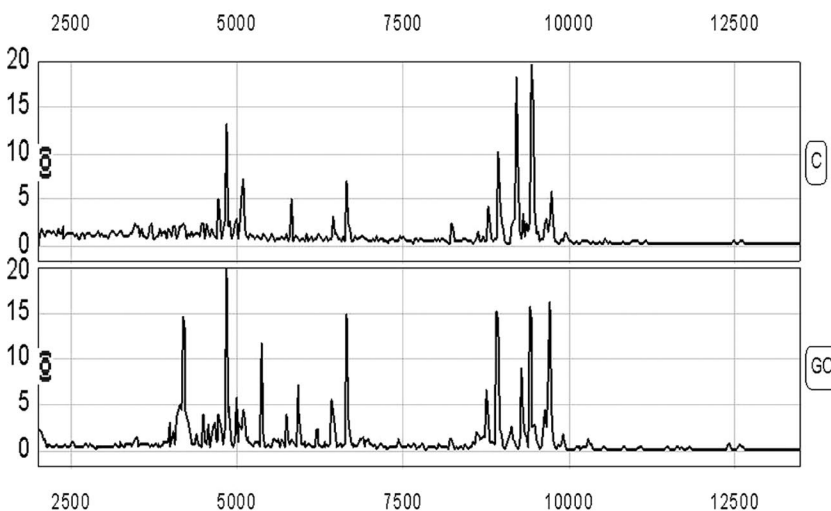

FIGURE 1. Representative protein spectrum of gallbladder cancer and control serum sample detected by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) combined with weak cation exchange (WCX) magnetic beads, showing the protein m/z between 2000 and 15,000.
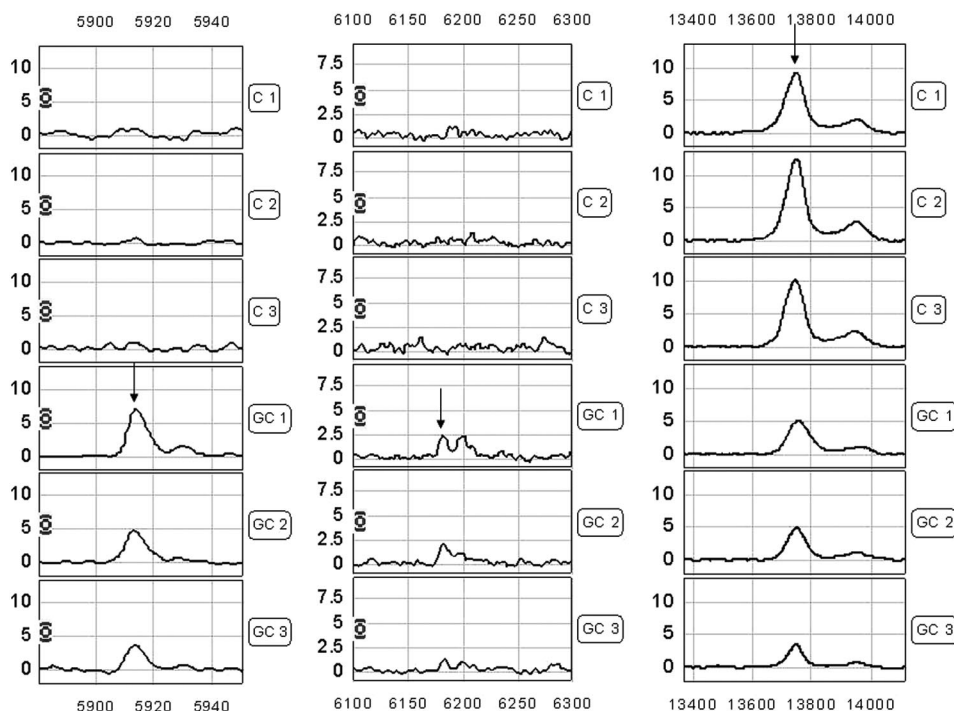
FIGURE 2. Differential expression of matrix-assisted laser desorption/ionization (MALDI) peak m/z 5913, 6181 and 13,752 in gallbladder cancer and control sera. Relative peak intensity is displayed along the *y* axis, and mass/charge ratios are shown on the *x* axis.

Da in the sera from patients with gallbladder cancer was significantly higher than that of the control group. The protein peak at 6181 Da in the sera from patients with gallbladder cancer was more abundant, when compared with normal healthy volunteers. Finally, the protein peak at 8691 Da in the sera from patients with gallbladder cancer was less abundant, when compared with the normal healthy volunteers (Table 2).

Identification of Biomarker Pattern and Construction of Diagnostic Model

The comparisons among different samples showed that the serum profiles from patients with cancer and control individuals were very similar despite a few of intersample variations. Therefore, the few variations that consistently differentiate these 2 different groups could be considered as potential disease biomarkers. We used the biomarker wizard function of the ProteinChip software to identify clusters of peaks differentially presented in gallbladder cancer serum samples compared with healthy controls. The 3 peaks (5913 Da, 6181 Da and 13,752 Da) with the highest discriminatory powers were automatically selected as the bases to construct a classification tree. Figure 3 showed the tree structure and sample distribution. The classification tree using the combination of the 3 peaks differentiated 30 gallbladder cancer and 30 healthy controls with a calculated sensitivity of 86.7% and a specificity of 93.3%. In

the blind test set, 18 of 20 true control cases were correctly classified, and 12 of 15 gallbladder cancer samples were correctly classified as malignant, suggesting a sensitivity of 80% and a specificity of 90% (Table 3).

DISCUSSION

Gallbladder cancer is a rare but lethal disease, taking up 0.8% to 1.2% in all cancers; however, it represents one of the most common duct tumors. The incidence of gallbladder cancer is fifth and for mortality rate is the ninth among all gastrointestinal tumors, with postoperative 5-year survival rate less than 5%. Gallbladder cancer occurs and develops without specific symptoms and is often clinically found in later stages, contributing to its high mortality rate. To improve early diagnosis and prognosis, the screening of specific tumor markers has been the hotspot nowadays. Some widely used markers include carbohydrate antigen 19-9, carcinoembryonic antigen, carbohydrate antigen 50, proliferating cell nuclear antigen, kinase inhibitor 67, epidermal growth factor receptor and transforming growth factor, among which carbohydrate antigen 19-9[6] and carcinoembryonic antigen[7] could be used for early diagnosis. Nevertheless, these markers exist in multiple tissue and organs, therefore lacking the specificity to diagnose gallbladder cancer.

MALDI-TOF MS is one useful tool for integrating separation and analysis of complex mixtures of proteins. Captured proteins are analyzed by TOF-MS, generating a spectral map depicting approximations of the molecular weights (m/z) and relative concentrations (intensity) of each protein (ion). When combined with WCX magnetic bead, it could capture more proteins in serum than strong anionic exchange magnetic beads, especially for the low molecular weight range. The technique has been extensively applied to tumor marker researches,[8,9] such as breast carcinoma,[10,11] nasopharyngeal cancer,[12] gastric cancer,[13] ovarian carcinoma[14] and so on.[15]

TABLE 2. Mean signal intensities of various proteins and peptides comparing gallbladder cancer with healthy control

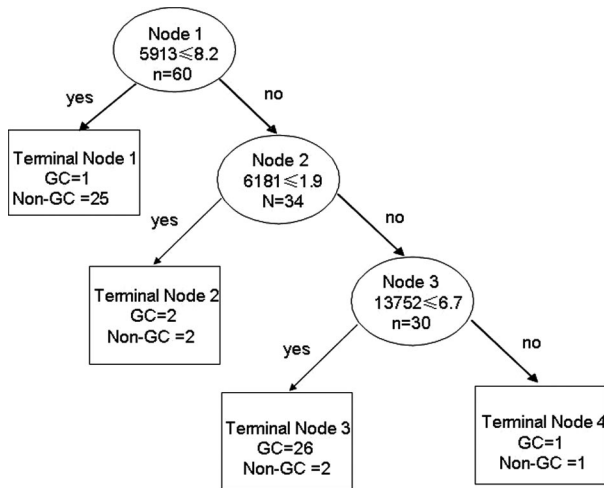| Protein mass-peak (m/z)	Gallbladder cancer	Healthy control	*P*
5913	4.32 ± 1.62	0.87 ± 0.41	4.2×10^{-7}
6181	1.68 ± 0.54	0.32 ± 0.10	2.6×10^{-6}
13,752	3.94 ± 1.06	8.65 ± 3.13	5.6×10^{-5}

FIGURE 3. The decision trees of diagnostic model for gallbladder cancer (GC) and non-GC (control). Each node was represented with different m/z value, and the diagnosis result went left or right depending on the detected peaks in the training test set. The root node (top) and descendant nodes were shown as ellipses, and the terminal nodes (nodes 1–4) were shown as rectangles. The mass value in the nodes was followed by lower or equal to intensity value. If the answer to the question in a node of the tree is yes, you proceed down to the left node, otherwise (ie, no), you proceed down to the right node. When proceeding to the terminal nodes, the decision tree assigned samples to 2 groups. Samples in terminal nodes 1, 2 and 4 were assigned to control and terminal node 3 was to gallbladder cancer. The numbers in rectangles represent the actual clinical diagnosis of samples assigned to this terminal node by decision tree (ie, in terminal node 1, decision tree assigned 21 samples to control but actually 20 of them were control according to the clinical diagnosis).

With MALDI combined with the WCX magnetic beads technology, we were able to analyze the protein profiles of 60 serum samples from patients with gallbladder cancer and healthy volunteers simultaneously. We identified potential biomarkers specific for gallbladder cancer, and 3 peaks 5913 Da, 6181 Da, 13,752 Da were chosen to set up the diagnostic model tree.[16,17] At node l, samples of m/z 5913 with peak intensities lower than or equal to 8.2 went to node 2, which had 5 healthy volunteers and 29 gallbladder cancer samples. Otherwise, samples entered terminal node 1, which had 25 healthy volunteers and 1 gallbladder cancer sample. At node 2, samples of m/z 6181 with peak intensities lower than or equal to 1.9 went to node 3, which had 3 healthy volunteers and 27 gallbladder cancer samples. The other samples entered terminal node 2,

which had 2 gallbladder cancer samples and 2 healthy volunteers. At node 3, samples of m/z 13,752 with peak intensities lower than or equal to 6.7 went to terminal node 3, which had 2 healthy volunteers samples and 26 samples of patients with gallbladder cancer. The other samples went to terminal node 4, which had 1 healthy volunteer sample and 1 gallbladder cancer sample. As presented in Table 3, the model identified 60 samples of the training set, 30 in gallbladder cancer and 30 in controls, with a sensitivity of 86.7% and specificity of 93.3%. In the blind test set, 18 of 20 true control cases were correctly classified, and 12 of 15 gallbladder cancer samples were correctly classified as malignant, yielding a sensitivity of 80% and a specificity of 90%.

In our diagnostic model, the 3 peaks with different m/z value might be specific biomarkers for gallbladder cancer or for some other disease. The increased candidate protein biomarker (5913 Da) was identified as internal fragment of fibrinogen alpha-E chain,[17] with theoretical mass of 5908 Da. It is also a positive marker in severe acute respiratory syndrome[18] and indicates relapse in gastric cancer.[19] The mass of our marker 3 (13,752 Da) is very similar to that of serum amyloid A as reported by Liu et al.[4] The other biomarker (6181 Da) still needs to be resolved in future studies. It should be noted that 1 m/z spot might represent many peptides with the same m/z ratio. The possibility is yet to be verified with high-performance liquid chromatography isolation of components of the single spots.

It should be noted that this study only compared groups with gallbladder cancer and health controls. In future studies, we recruit more controls with other gallbladder diseases or other cancers to improve the specificity of our reported biomarkers in present study.

CONCLUSION

The serum proteome profiles of gallbladder cancer were generated with the combination of MALDI-TOF MS and WCX magnetic beads and pattern recognition software in our study. We have showed that the power of proteomics approaches in the discovery of new biomarkers, which would provide a rapid and accurate mode of analysis for the detection of multiple disease-related proteins simultaneously, reproducibly and in high-throughput manner. With the panel of 3 selected biomarkers, we could achieve high sensitivity and specificity for the detection of gallbladder cancer. We expect to explore the structure and function of these protein biomarkers for gallbladder cancer development/progression in future studies.

TABLE 3. The prediction results of the diagnostic model for gallbladder cancer

Group	Samples	Cases	Correct classed	Accurate %
Training set	Gallbladder cancer	30	26	86.7
	Control	30	28	93.3
Blinding set	Gallbladder cancer	15	12	80
	Control	20	18	90

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