Screening and identification of serum proteomic biomarkers for gastric adenocarcinoma

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Abstract. The aim of the present study was to screen for possible serum biomarkers for gastric adenocarcinoma. Surface-enhanced laser desorption ionization time of flight mass spectrometry (SELDI-TOF-MS) was used to screen serum samples from 109 cases of gastric adenocarcinoma and 106 control subjects (60 healthy subjects, 30 patients with chronic superficial gastritis and 16 cases of chronic atrophic gastritis). The differentially expressed protein peaks were selected and isolated using high performance liquid chromatography (HPLC) and processed with enzyme prior to liquid chromatography-mass spectrometry tandem mass spectrometry (LC-MS/MS) analysis and data mining with software XCalibur program components BioWorks 3.2. Among the gastric cancer cases, three differentially expressed protein peaks were selected as potential serum biomarkers: the m/z peaks at 5,906.5 showed increased expression (8.53±4.33 in the cancer group, and 0.88±0.31 in the control group); the m/z peaks at 6,635.7 and 8,716.3 showed decreased expression (6.54±2.44 and 0.93±0.29, respectively, in the cancer group and 17.56±4.43 and 2.16±0.98, respectively, in the control group) ($P<0.01$). The m/z peaks at 5,906.5, 6,635.7 and 8,716.3, were identified as fibrinogen α-chain, apolipoprotein A-II and apolipoprotein C-I. The combined use of the three biomarkers distinguished the cancer group patients from the control group samples at a sensitivity of 93.85% (61/65) and a specificity of 94.34% (50/53). In conclusion, fibrinogen α-chain, apolipoprotein A-II and apolipoprotein C-I were identified as potential markers for gastric cancer and appear to have diagnostic value for clinical applications.

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Introduction

Gastric adenocarcinoma is a common gastrointestinal malignant tumor, accounting for 23.2% of cancer-related deaths in China (approximately 16 million individuals) (1,2). At present, the clinical diagnosis of gastric adenocarcinoma mainly relies on physical and histological examinations, which are accurate only for middle- or late-stage cases. Therefore, many patients are diagnosed at a late stage of the disease. Biochemical markers such as carcinoembryonic antigen, carbohydrate antigen 19-9 and carbohydrate antigen 125 are used as markers for diagnosis; however they are non-specific and lack adequate sensitivity (3-6). The present study aimed to design a new diagnostic system with high sensitivity and specificity for early-stage gastric cancer detection. We employed a newly emerging technique, surface-enhanced laser desorption ionization time-of-flight mass spectrometry (SELDI-TOF-MS) (7-12), to analyze the serum proteome from healthy volunteers, gastric cancer patients and gastritis patients, and to screen for specific protein biomarkers for gastric cancer (13-15).

Materials and methods

Clinical data. One hundred and nine gastric cancer patients (males 41, females 68, age range 32-89 years) (25 cases Dukes' A, 22 cases Dukes' B, 28 cases Dukes' C and 34 cases of Dukes' D), and 106 cases of controls (males 50, females 56, age range 26-85 years) (60 healthy volunteers, 16 cases of chronic atrophic gastritis patients, and 30 cases of chronic superficial gastritis) were recruited for this study at the First Affiliated Hospital of Zhejiang University and Zhejiang Taizhou Municipal Hospital. The subjects were assigned into an experimental group and a verification group according to Table I. The peripheral blood samples were collected in the morning after overnight fasting. The blood samples were then maintained at 4˚C for 1-2 h prior to centrifugation at 3,000 rpm at 4˚C for 10 min to separate out the serum. The serum samples were frozen at -80˚C in a freezer for storage. All protocols and experiments were approved by the Taizhou Medical College Ethics Committee for clinical experiments and use of human samples; written informed consent was obtained from all subjects participating in this study. The study complied with the World Medical Association Declaration of Helsinki regarding ethical conduct of research involving human subjects.

Table I. Clinical subjects involved in the study.

Groups	Experimental	Verification	Total
Gastric adenocarcinoma			
Dukes' A	15	10	25
Dukes' B	12	10	22
Dukes' C	20	8	28
Dukes' D	18	16	34
Control			
Chronic superficial gastritis	15	15	30
Chronic atrophic gastritis	8	8	16
Healthy controls	30	30	60
Total	118	97	215

Protein chip analysis. After thawing and 10 min of centrifugation (10,000 rpm), a 20-μl serum sample without fraction treatment (which does not affect the protein mining efficiency as shown by the authors) was added to 30 μl 0.5% U9 (9 mol/l urea, 2% CHAPS (3[(3-cholamidopropyl)dimethylammonio]-l-propanesulfonate), 1% DTT (DL-dithiothreitol)) in a 96-well plate and incubated for 20 min at 4˚C with 600 rpm vigorous agitation. The ProteinChip array cassette was put into a 96-well bioprocessor and 100 μl U1 buffer (50 mmol/l Tris-HCL diluted 10% U9 buffer) was added into each well, and incubated for 10 min at 4˚C with 600 rpm vigorous agitation. Q10 buffer (200 μl) (100 mM Tris-HCl buffer pH 9.0) was then added and a 5-min incubation was carried out 2 times with agitation. All experimental reagents were obtained from Shanghai Shenggong Company, Shanghai, China.

Fifty microliters of the protein-denatured serum samples were removed to a new tube, and 200 μl Q10 buffer was added to dilute the samples before being applied onto the Q10 chip Bioprocessor (Ciphergen) for 60 min. Then each plate of the Q10 chip was added together with 200 μl Q10 buffer, incubation was carried out for 5 min two times with agitation, and finally 20 mm/l HEPES (pH 7.4) buffer was added for washing before drying. SPA (0.5 μl) was added 2 times into each plate with drying between each addition. Mass spectrometry was set as laser intensity 185, sensitivity 8, 2,000-20,000 m/z. Inter-chip CV was <10%. All-in-one control chip was used to adjust the system with a systemic error <0.1%. All of the data were processed with ProteinChip 3.0 software, then the Biomarker Wizard software 3.1 and Biomarker Wizard software 4.0.1. P<0.01 was determined to indicate statistically significant differences in the comparison between two protein peaks.

Purification and identification of specific protein peaks. Serum samples (100 μl) with 300 μl water and 700 μl acetonitrile were mixed and maintained at -20˚C in a freezer for 30 min prior to centrifugation at 3000 rpm for 10 min. The supernatant was freeze-dried for 20 min before collection for HPLC. The purified solutions were collected at different time periods, freeze-dried to obtain a 20-μl volume solution. Solution (0.5 μl) was mixed with 1.5 μl matrix solution (10 mg/ml CHCA) to be placed on chip points, with crystallization for MALDI-TOF MS detection. The conditions included pulsed nitrogen laser (337 nm), accelerating voltage 20 KV, linear analysis mode, mass range 3,000-20,000 m/z. The samples corresponding to the specific protein peaks in SELDI-TOF MS were subsequently identified.

Table II. Intensity of the three differential peaks in gastric adenocarcinoma and control groups.

(m/z)	Gastric adenocarcinoma	Control	P-values
5,907.5	8.53±4.33	0.88±0.31	2.8×10^{-7}
6,636.7	6.54±2.44	17.56±4.43	4.5×10^{-6}
8,716.3	0.93±0.29	2.16±0.98	8.4×10^{-4}

LC-MS/MS analysis. Purified target protein of 20 μl was mixed with 60 μl 8M urea (final concentration of urea 6 M) and was agitated at room temperature for 20 min. Then 0.8 μl 1 M DTT (final concentration 10 mM) was added and mixed at room temperature for 1 h, and 3.2 μl 1 M iodine acetyl amine (final concentration 40 mM) was added and maintained for 45 min in the dark. DTT (3.2 μl 1 M) (final concentration 40 mM) was added for 20 min, and then 400 μl 50 mM NH_4HCO_3 was added to dilute the solution, with urea concentration at 1 M and pH 8.0. Subsequently, 0.1 μg protease in a 37˚C water bath for 1 h, and formic acid was adjusted to a pH <3 to terminate the reaction. The hydrolysates were subjected to LC-MS/MS analysis. Sample solutions were put in a self-made C18 capillary column for liquid chromatography: inner diameter 100 μm, filled part 100 mm, filled particles with diameter 5 μm. The flow phase A was water and 0.1% formic acid; the flow phase B was acetonitrile and 0.1% formic acid. The washout followed the sequences below: 100% A (0 min) - 100% A (5 min) - 5% B (5.1 min) - 65% B (60 min) - 100% B (75 min) - 100% B (85 min). The flow speed was 200-800 nl/min. The data-dependent mode was used; the scanning ranges were from 400 to 2,000 m/z; the five strongest signal peaks of each full scan were selected for secondary MS (MS2) analysis.

The data retrieval used the XCalibur program components BioWorks 3.2 (Thermo Finnigan) and the peptide sequences were searched for in the NCBI human protein database (human.ref) according to the mass spectrum. The following parameters were used: enzyme split site at random site; fixed modification at cysteine amine formylation modification; variable modification at methionine oxidation; retrieval parameters ΔCN >0.1; Sp >500; Rsp ≥5; Xcorr vs. Charge: Xcorr (+1) >1.9, Xcorr (+2)>2.5, Xcorr (+3) >3.75.

Results

The serum protein fingerprint spectrum. The protein fingerprint spectrum from 65 cases of gastric adenocarcinoma and 53 cases of control were normalized first, then analyzed using Biomarker wizard software. Two hundred and twenty-seven protein peaks were found in the m/z range from 2,000-50,000 (Fig. 1).

Data analysis of the gastric adenocarcinoma protein fingerprint spectrum and the diagnostic model. The Biomarker Wizard software analysis revealed three differentially expressed

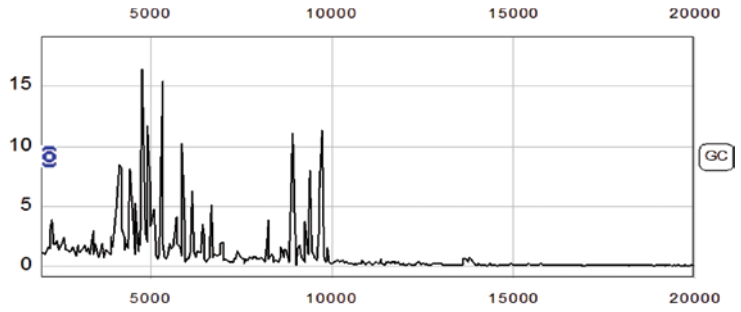

Figure 1. Serum proteomic spectrum of the gastric adenocarcinoma patients.

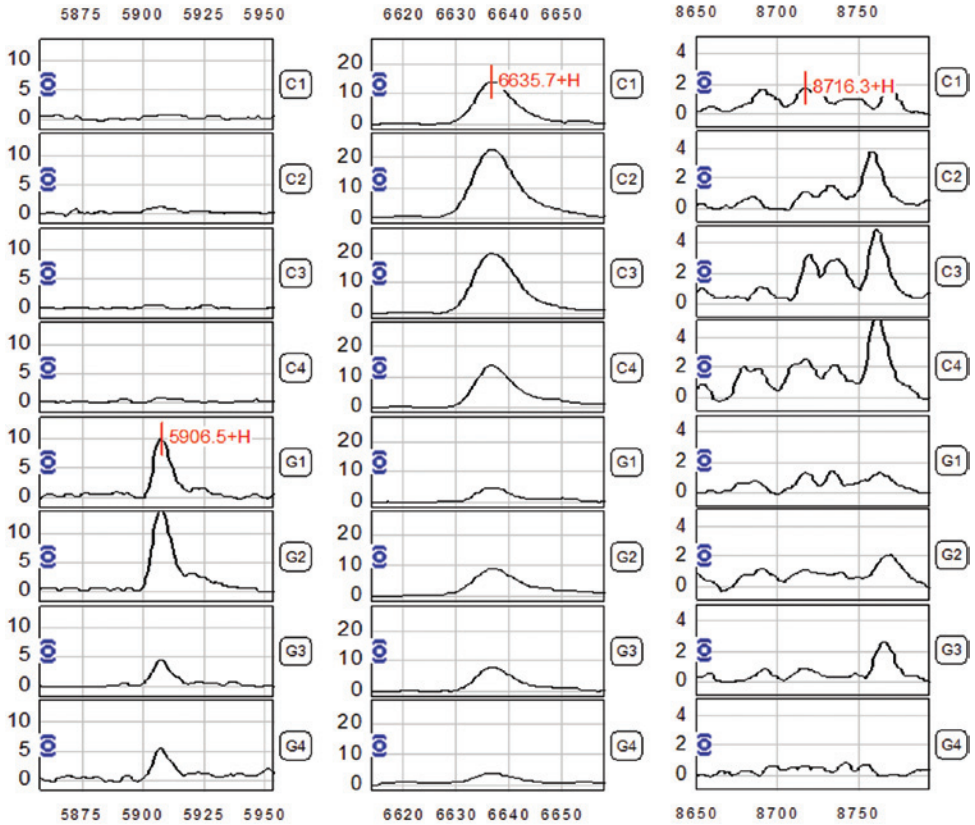

Figure 2. Protein fingerprint of the three protein peaks (m/z 5,906.5, 6,635.7, 8,716.3) for the diagnostic model of the gastric adenocarcinoma cases.

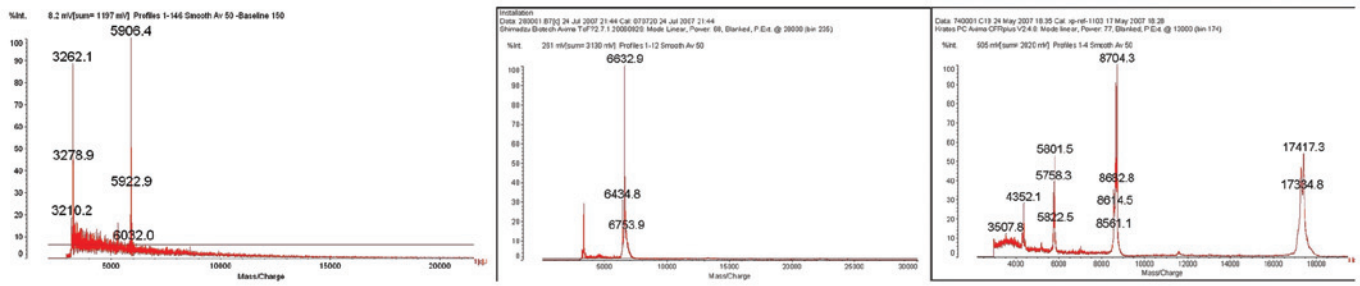

Figure 3. MALDI-TOF image of the three differential protein peaks (m/z 5,906.5, 6,637.6, 8,716.3).

protein peaks in serum samples from gastric adenocarcinoma patients, with m/z at 5,906.5, 6,635.7 and 8,716.3 respectively (P<0.01) (Fig. 2 and Table II), which were considered as potential biomarkers for gastric adenocarcinoma. The 5,906.5 peak showed increased expression in the gastric adenocarcinoma cases; while the 6,635.7 and 8,716.3 peaks showed a decreased

Table III. Characteristics of the gastric adenocarcinoma diagnostic model.

Set	Groups	Case number	Correct cases	Diagnosis rate (%)
Experimental	Gastric adenocarcinoma	65	61	93.85
	Control	53	50	94.34
Verification	Gastric adenocarcinoma	44	40	90.91
	Control	53	48	90.57

Table IV. Amino acid sequence of the differential protein peaks in the gastric adenocarcinoma cases.

(m/z)	Protein	Molecular weight	Amino acid sequence	Match rate (%)
5,906.5	Fibrinogen α-chain	5,904.0	SSSYSKQFTS STSYNRGDST FESKSYKMAD EAGSEADHEG THSTKRGHAK SRPV	100
6,635.7	Apolipoprotein A-II	6,630.0	TPDY SSALDKLKEF GNTLEDKARE LISRIKQSEL SAKMREWFSE TFQKVKEKLK IDS	96
8,716.3	Apolipoprotein C-I	8,707.8	QAK EPCV ESLVSQYFQT VTDYGKDLME KVKSPELQAE AKSYFEKSKE QLTPLIKKAG TELVNFLSYF VELGTQPATQ	60

expression level in the gastric adenocarcinoma serum samples (Table II). The combined analysis with the three peaks as the basis for the diagnostic model showed a sensitivity of 93.85 (61/65) and a specificity of 94.34% (50/53) in analyzing the mass spectrometry data from the 65 gastric adenocarcinoma patients and 53 cases of control subjects (Table III).

Purification of the protein peaks and the MS analysis. The protein peaks of the three biomarkers (m/z 5,906.5, 6,635.7 and 8,716.3) were isolated and purified with HPLC, and then collected into PCR tubes for MALDI-TOF-MS examination. The results showed that the three peaks were proteins with molecular weights of 5,906.4, 6,632.9 and 8,704.3, respectively (Fig. 3).

Following LC-MS/MS measurement of the digested proteins and the data screening from NCBI human protein database, the 5,906.4 peak was found to correspond to the fibrinogen α chain (100% match), the 6,632.9 peak corresponded to the apolipoprotein A-II (96% match), and the 8,704.3 peak corresponded to the lipid-laden protein C-I (60% match) (Table IV).

Discussion

Gastric cancer is a cancer without clear symptoms at onset, and metastasis and recurrence are common (16,17). The prognosis of this disease is poor as well. Early diagnosis of gastric cancer urgently requires novel techniques with high sensitivity and specificity. Since the onset and progression of cancer lead to characteristic changes in the serum proteome, it is possible to employ proteomic techniques to screen for potential biomarkers of gastric cancer (11,18,19). The present study utilized SELDI-TOF MS, and successfully identified a new diagnostic model for gastric cancer, including suitability for early-stage patients.

SELDI-TOF MS is an ideal platform for proteomic studies with several advantages. i) A small amount of sample is required. The scan is fast and suitable for clinical diagnosis and high throughput-screening analysis. ii) The technique can identify the specific spectrum including several biomarkers at the same time. iii) Crude samples without prior purification can be used. iv) The technique can be combined with many genomic techniques. v) The technique is of high reliability and can be reproduced in repeated tests. vi) The technique is applicable to proteins that are not suitable for 2D-PAGE analysis, such as those with extremely small molecular weights, or hydrophobic, transmembrane, as well as isoelectric point (8-10). The technique currently shows progressive results in biomarker screening of autoimmune diseases, inflammation disorders and many types of malignant cancers (9,10,20-24), providing the basis for diagnosis and treatment of these diseases clinically.

The present study described three potential biomarkers for gastric cancer with high sensitivity and specificity in both the experimental and verification set, as mentioned in Results. The three identified markers were fibrinogen α-chain, apolipoprotein A-II, and lipid-laden proteins C-I. They may play different roles in the onset and progression of gastric cancer. Fibrinogen participates in blood coagulation processes, and it may mediate the interaction of cancer cells and platelet, which occurs during cancer metastasis (25,26). Apolipoprotein participates in lipid transport and may be involved in cell proliferation/apoptosis regulation (27,28). C-I is mainly synthesized in the liver, and is less well-known in cancer biology. Several previous studies have shown a lower expression of C-I in serum from cancer patients (29,30), which was consistent with the present study. However the detailed mechanism requires future studies.

Taken together, the present study proved the efficiency of SELDI-TOF MS in screening for biomarkers of gastric cancer in a serum proteome-based manner. The three discovered biomarkers could be effectively used for gastric cancer diagnosis. Due to the limited number of patients, we did not perform a correlation analysis between the stage of cancer progression and the biomarker profiles. It is necessary to recruit more patients with early-stage disease to identify various biomarkers for the diagnosis of patients as early as possible.

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