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Full Length Research Paper

Serum protein fingerprint of patients with gastric cancer by SELDI technology

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To study the serum protein fingerprint of patients with gastric cancer and to screen for protein molecules closely related to gastric cancer during the onset and progression of the disease using surface-enhanced laser desorption and ionization time-of-flight mass spectrometry (SELDI-TOF-MS). Serum samples from 80 gastric cancers and 80 healthy volunteers. WCX2 protein chip and PBSII-C protein chips reader (Ciphergen Biosystems Ins.) were used. The protein fingerprint expression of all the serum samples and the resulting profiles between cancer and normal were analyzed with Biomarker Wizard system. A group of proteomic peaks were detected. Four differently expressed potential biomarkers were identified with the relative molecular weights of 5907, 8678, 11673 and 13725 Da. Among them, two proteins with m/z 8678 and 13725 Da down-regulated, and two proteins with m/z 5907 and 11673 Da were up-regulated in gastric cancers. This diagnostic model can distinguish gastric cancer from healthy controls with a sensitivity of 96% and a specificity of 93.3%. SELDI technology can be used to screen significant proteins of differential expression in the serum of gastric cancer patients. These different proteins could be specific biomarkers of the patients with gastric cancer in the serum and have the potential value of further investigation.

Key words: SELDI, gastric cancer, biomarker, proteomics.

INTRODUCTION

The early detection of cancer is crucial for its ultimate control and prevention. Most of today's licensed tests for disease detection are protein-based assays. Technologies such as multidimensional separation systems directly coupled to mass spectrometry analysis represent improvement in sensitivity and throughput when compared with traditional two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) analysis for biomarker discovery (Linke et al., 2007). Proteomic pattern diagnostics combines proteomic pattern profiling of tissue and body fluids by mass spectrometry with sophisticated bioinformatics tools to identify patterns within the complex proteomic profile that discriminate between normal and disease states. Proteomic pattern diagnostics has been successfully applied to solve the problems of early detection for a number of different types of cancer (Cho, 2006, 2007; Wulfkuhle et al., 2003; Cho et al., 2007).

Gastric cancer is a debilitating disease associated with a high mortality. Its successful treatment relies on an early diagnosis, but this remains a challenge since the progression of the malignancy is usually silent until it reaches a more advanced stage where the prognosis is poor. Certainly, early detection can drastically facilitate treatment and improve the long-term survival of the patient (Kodera et al., 2003; Leung and Sung, 2002).

The sensitivity of the current single biomarkers in tumor diagnosis is low (usually less than 40%) and complicated by a high return of 'false-positives'. Further, none of the existing serum markers can be used individually for screening for gastric cancer. It would be highly desirable to have a new rapid and sensitive diagnostic test for gastric cancer (Schneider et al., 2005; Schneider et al., 2005).

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Abbreviations: SELDI, Surface-enhanced laser desorption and ionization; **SELDI-TOF-MS,** surface-enhanced laser desorption and ionization time-of-flight mass spectrometry; **CEA,** carcinoembryonic antigen; **SARS,** severe acute respiratory syndrome; **CV,** co-variance; **2D-PAGE,** two-dimensional polyacrylamide gel electrophoresis; **WCX2,** ciphergen weak cation exchange; **BPS,** biomarker pattern software.

Table 1. Serum samples used in training and testing sets.

Samples	Training set	Testing set	Total
Gastric cancer	50	30	80
Healthy volunteers	50	30	80
Total	100	60	160

In this study, we aimed to search differentially expressed proteins as potential biomarkers in gastric cancer patients by surface-enhanced laser desorption-ionization time-of-flight mass spectrometry (SELDI-TOF-MS). We used Ciphergen weak cation exchange (WCX2) protein chip to screen potential serum biomarkers for gastric cancer detection. A total of 160 serum samples from gastric cancer patients or healthy volunteers were collected and analyzed simultaneously. A panel of differentially expressed proteins was advocated for biomarkers of diagnosis for gastric cancer.

MATERIALS AND METHODS

Patients and volunteers

Experiment was performed in Taizhou Municipal Hospital, Zhejiang, China in May 2008. Studies were conducted using Chinese patients diagnosed with gastric cancer (invasive gastric adenocarcinoma, n = 80) at Taizhou Municipal Hospital, Zhejiang, China. Comparative studies were also performed using healthy volunteers (n = 80). The studies were approved by the local Ethics Committee of Taizhou Municipal Hospital, and had the informed consent of the patients and volunteers. The patients and serum samples were then divided into two groups: the “training” set and the blinded “test” set (Table 1).

Sample collection

Approximately 5 ml of blood was withdrawn via vein puncture from each patient and serum prepared and stored at -80°C prior to analysis.

ProteinChip analysis

Serum specimens were thawed on ice, followed by centrifugation at 20,000 rpm for 10 min, and prepared for analysis on WCX2 chips (Ciphergen Biosystems, Inc., Fremont, USA). In brief, WCX2-arrays were equilibrated with 150 µl, 100 mM sodium acetate with pH 4.0 and containing 0.02% Triton X-100 for 5 min. The binding/ washing buffer was then rinsed and replaced with 150 µl of fresh binding buffer plus 20 µl of serum and incubated on a reciprocating shaker for 20 min. The chips were then washed with distilled water to remove salts before allowing them to air-dry for 15 min. 0.5 µl of saturated sinapinic acid solution (40 % v/v acetonitrile and 0.5 % v/v trifluroacetic acid) was added twice to each spot. After drying, samples were analysed using an automated protocol in a deep-well type protein chip reader (PBS II-C, Ciphergen Biosystems, Inc., Fremont, USA) via PBSII-C SELDI-MS, with a nitrogen laser (337 µm) utilizing 185 µJ as the upper energy limit.

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), hirudin (7033.6 Da) (Ciphergen Biosystems, Fremont, CA, USA).

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

RESULTS

Detection of the protein peaks

Proteomic data from the samples of the training set (consisting of 50 gastric cancer patients and 50 controls) were analyzed with Biomarker Wizard software 3.1. Up to 163 protein peaks per spot were detected between m/z 1000 and m/z 50000 and it showed the effectiveness of the SELDI technology separation of low molecular weight proteins (< l 5000) (Figure 1).

Optimization of the experimental conditions and evaluation of the reproducibility

Reproducibility was evaluated with four mixed serum samples from the healthy controls of blood type O (two women and two men). The mixed serum samples were spotted on a four spot WCX2 protein chip. The co-variance (CV) was under 10% for all the selected mass

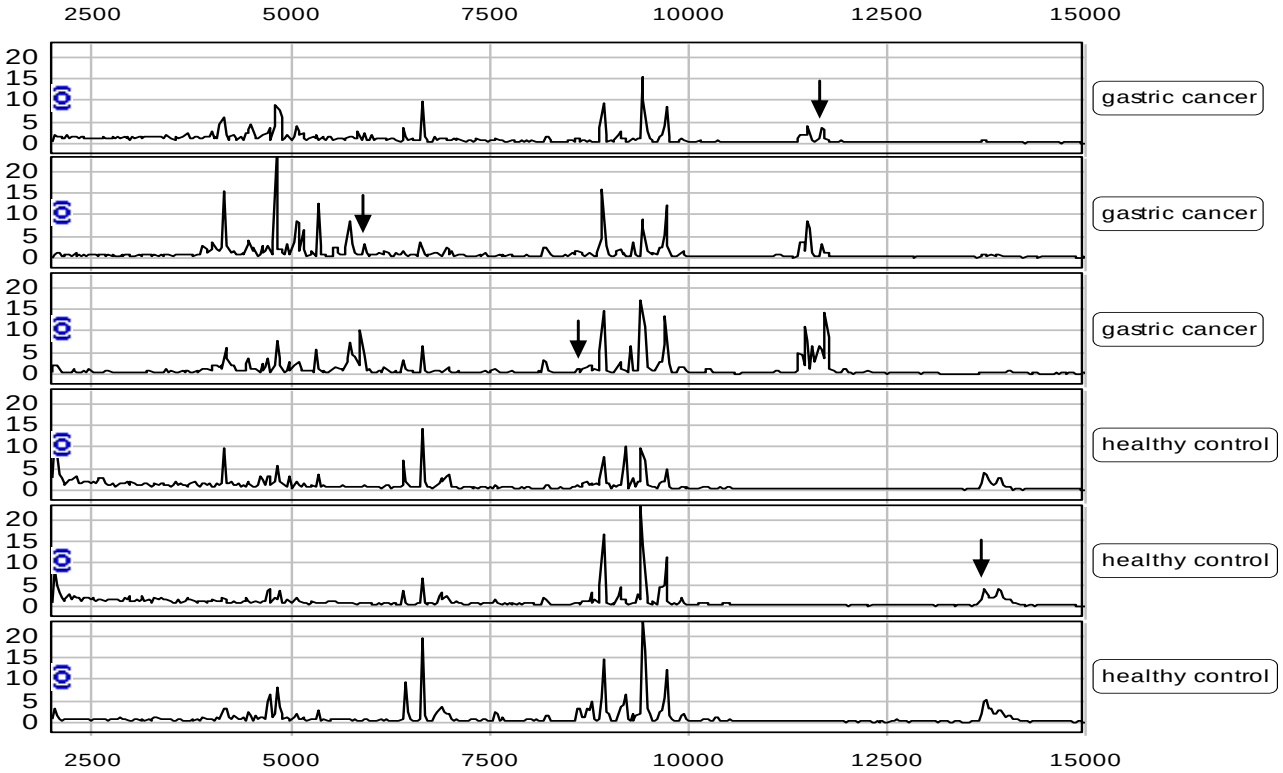

Figure 1. Protein peaks of patients with gastric cancer and healthy control group after standardization.

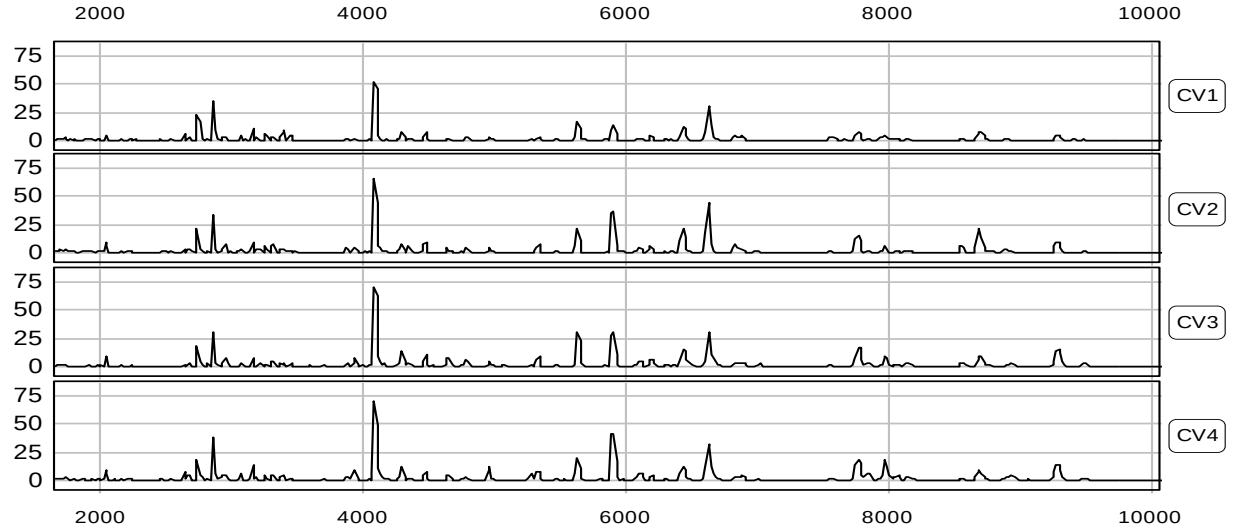

Figure 2. A 4-spot reproducibility test showed good reproducibility. The CV of all the selected mass peaks was below 10%.

peaks (Figure 2).

Identification of biomarker pattern and construction of diagnostic model

The comparison among different samples showed that the serum profiles from cancer patients and control individuals were very similar in spite of a few of inter-sample variations. Therefore, the few variations that consistently differentiate these two different groups could be considered as potential disease biomarkers. Here, we used the biomarker wizard function of the ProteinChip software to identify clusters of peaks differentially presented

Table 2. Intensities of the 5907, 8678, 11673 and 13725 m/z peaks.

Peaks (m/z)	intensities of arbitrary units($\bar{x}$ ± s)		p value
	cancer	control	
5907	7.56 ± 3.71	1.42 ± 0.95	3.1×10^{-7}
8678	3.57 ± 4.17	11.02 ± 11.85	3.1×10^{-5}
11673	14.48 ± 7.52	2.27 ± 2.93	3.1×10^{-6}
13725	1.36 ± 1.99	6.64 ± 7.48	3.1×10^{-5}

in gastric cancer serum samples compared with the control serum samples; we obtained 32 discriminating protein peaks in sera. To develop biomarker patterns for the diagnosis of gastric cancer, the intensities of the protein peaks in the training set were submitted to BPS. A total of four peaks (5907, 8678, 11673 and 13725 Da) with the highest discriminatory power were automatically selected to construct a classification tree. Peak 5907 and 11673 Da were up-regulated in gastric cancers. For the peak with a mass of 5907 Da, significantly higher normalized intensity was observed in the gastric cancer group compared with the control group. The second peak of mass 11673 Da showed the same distribution with a normalized intensity of 14.48 ± 7.52 for the gastric cancer group and 2.27 ± 2.93 in the control group. In addition, peak 8678 and 13725 Da were down regulated in gastric cancers. The normalized intensity of peak 8678 was observed in the gastric cancer group 3.57 ± 4.17 compared with the control group 11.02 ± 11.85 and the peak of mass 13725 Da with a normalized intensity of 1.36 ± 1.99 for the gastric cancer group and 6.64 ± 7.48 in the control group (Table 2 and Figure 3). The classification tree using the combination of the four peaks identified 50 gastric cancer and 50 healthy subjects with a calculated sensitivity of 96% and a specificity of 93.3%.

Test of the diagnostic model for PBC in a blind test

Sixty samples, including 30 from patients with gastric cancers and 30 healthy controls (Table 1) were used to test the gastric cancer diagnostic model. The classification tree discriminated the gastric cancer samples from the controls with a sensitivity of 92% and a specificity of 90%.

DISCUSSION

In recent few years, it has become possible to find new tumor markers for diagnosing and monitoring the occurrence and development of tumors as the proteomics research has been developed (Rodland, 2004). SELDI technology is a new protein separation and identification technology developed in recent years. It is simple, rapid, high-throughput, and needs small amount of samples, and particularly, it can capture proteins of small molecular weight and low abundance more effectively (Issaq et al., 2002). It has been extensively applied to the researches about tumor markers (Conrad et al., 2004; Petricoin and Liotta, 2004), such as prostate cancer (Walsh, 2003; Malik et al., 2005), breast carcinoma (Zeidan and Townsend, 2008), bladder cancer (Langbein et al., 2006), hepatocellular carcinoma (Paradis et al., 2005), naso-pharyngeal cancer (Cho et al., 2004) and so on (Kozak et al., 2003).

The relative molecular weight of the representative specific proteins (m/z 5907, 11673, 867 and 13725) found in patients with gastric cancer in our experiment; using SELDI technology, is all small. Their properties are not clear for the moment, and we speculate they may be specific polypeptides or specific protein fragments that contain 40 to 70 amino acids (probably proteins and peptides secreted by tissue cells or tumor cells or their metabolites, or soluble membrane antigen). Because these proteins have low molecular weight, and their content is small, we cannot detect them previously by conventional methods (Adam et al., 2002; Issaq, 2001). The sensitivity and specificity of the current commonly used clinical markers such as serum carcinoembryonic antigen (CEA, molecular weigh 150300 Da), and glycoprotein antigen (CA199 molecular weight 5000 kD), are very poor. The sensitivity for early gastric cancer is low. And the positive rate for incipient tumors whose diameter is smaller than 2 cm is only 37.5%. The tumor cells in the earlier period of patients gastric cancer are confined to the mucosal tissue, and do not invade surrounding vessels or tissues, so the proteins and polypeptides secreted by tumor cells or produced by metabolism of tumor cells can not be detected. Theoretically, the macromolecule proteins are not easy to infiltrate into the blood, but the micromolecule proteins are easy to enter the blood circulation. This provides support for finding these small molecule proteins, as the sensitivity of the SELDI is up to 1 mol and it can help to detect certain protein changes in tumors at the early time. So theoretically, this technology is feasible for early warning for tumors, and the advantage of the SELDI technology is to detect proteins of low molecular weight and low peaks.

The complex changes of serum proteomics reflect the metabolism states of the physiological and pathological organ (Latterich et al., 2008; Hanash, 2003). In the study,

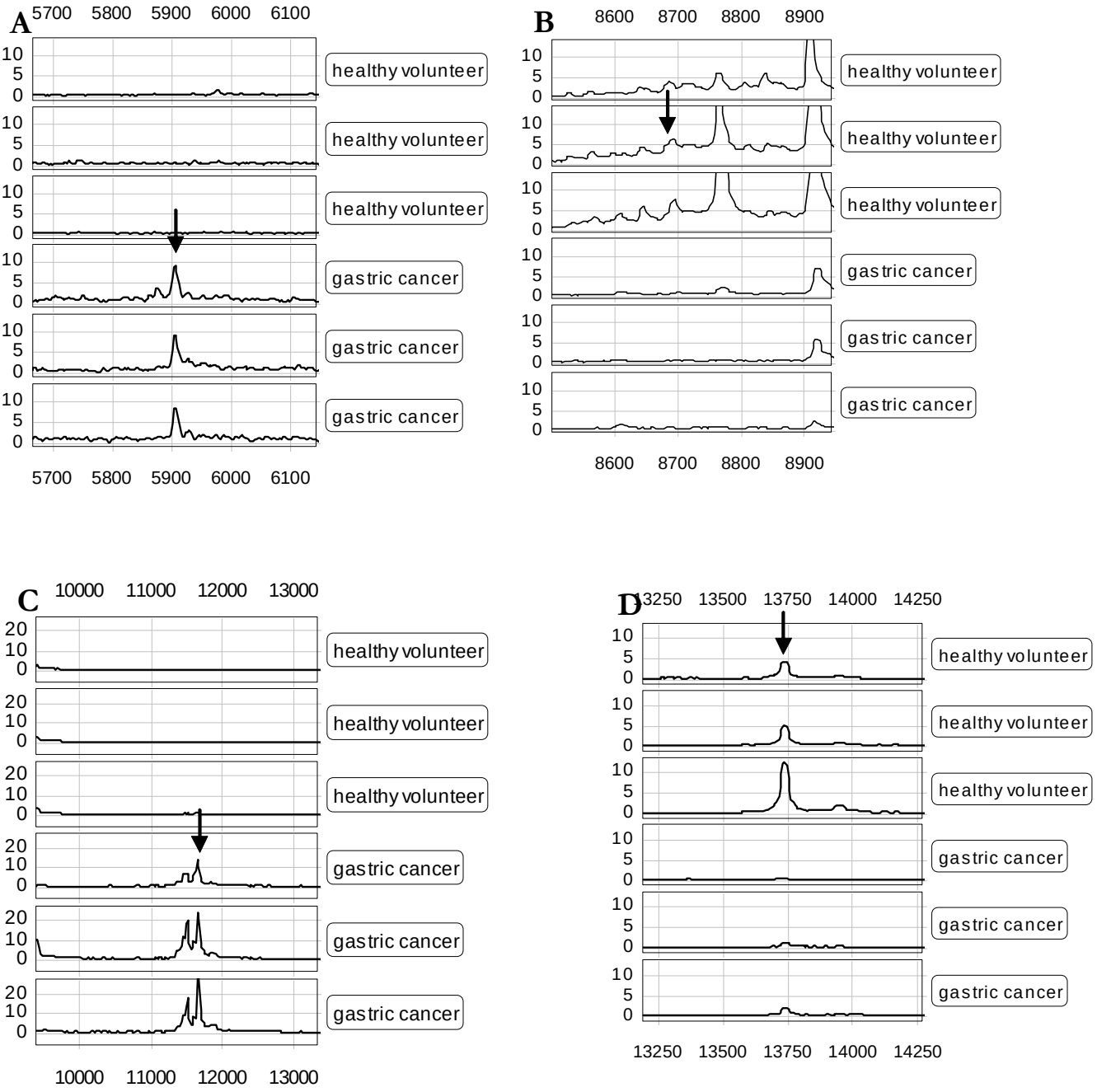

Figure 3. Differential expression of surface-enhanced laser desorption and ionization peak m/z 5907, 8678, 11673, 13725 in gastric cancer (bottom 3) and control (top 3) sera. A: (5907Da) Up-regulated in gastric cancer. B: (8678Da) Down-regulated in gastric cancer. C: (11673Da) Up-regulated in gastric cancer. D: (13725) Down-regulated in gastric cancer.

we found that m/z 5907 and 11673 in the serum of patients with gastric cancer were significantly higher than those in control group, which are speculated to come from the relevant pathological tissues or related reaction, while m/z 8678 and 13725 in the serum of patients with gastric cancer were significantly lower than those in control group, which is speculated to have two possibilities: One is that the special biology characteristics of tumors causes consumption state of cachexia, which results in the decline of various proteins such as albumin. Another possibility is that they are proteins or peptides synthesized by tumor suppressor gene. The classification tree using the combination of the four peaks identified 50 gastric cancer and 50 healthy subjects with a calculated sensitivity of 96% and a specificity of 93.3%. In our blind test, it demonstrated good sensitivity and specificity: 92

and 90%, respectively. These results indicate that useful serum biomarkers for gastric cancer can be discovered by SELDI-TOF-MS combined with the use of protein chip and pattern recognition software. The pattern of multiple markers provides a powerful and reliable diagnostic method for gastric cancer with high sensitivity and specificity.

Compared with similar studies abroad (Bhattacharyya et al., 2004; Koopmann et al., 2004; Yu et al., 2005; Honda et al., 2005; Ehmann et al., 2007), the candidate markers we found are not the same, especially the down-regulated protein peaks. The reason, on the one hand, may be that our detection method, control of serum quality, the equipment standardized state and automation applications were not unified. On the other hand, it may be caused by the difference in racial genetic factors and diet habits or it may be due to the fact that most of our patients were in the mediate or late state, while the patients aboard were mainly in the early state.

In our diagnostic model, the four peaks may be biomarkers unique for gastric cancer or for some other disease. Liu et al. (2009) identified an 11.6 kDa positive protein marker derived from serum amyloid A; its theoretical mass is 11.68 kDa, and the mass of our marker 3 (11.67 kDa) is very similar to that of serum amyloid A. It is a positive marker in severe acute respiratory syndrome (SARS) (Ren et al., 2004) and indicates relapse in nasopharyngeal cancer (Cho et al., 2004). Serum amyloid A is an acute phase protein that is associated with circulating high-density lipoproteins and that modulates lipid trafficking and immune responses. It is also the precursor protein in reactive amyloidosis (Dan et al., 2006). This information will help us in further investigations.

In the SELDI technology, each m/z value may represent many peptides of similar molecular weight, so we can not identify the proteins in the body fluid. Therefore, we can not get the structures, functions of the proteins, and it needs to be resolved in next studies (Cho et al., 2007).

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