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# Detection of Elevated Proteins in Peritoneal Dissemination of Gastric Cancer by Analyzing Mass Spectra Data of Serum Proteins

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***Background.*** **Recently, several authors reported on the Protein Chip approach to analyze serum. They used SELDI-TOF-MS (surface enhanced laser desorption/ionization-time of flight-mass spectrometry) to identify patients with cancers of various origins in a highly sensitive and specific manner. In the current study, a similar approach was employed to analyze the serum of patients with various stages of gastric cancer.**

***Methods.*** **Control serum specimens from patients with gastritis ($n = 19$) and those with gastric cancer (Stage I: $n = 6$, Stage II or III: $n = 6$, Stage IV: $n = 6$, total: $n = 18$) were collected and analyzed by the Protein Chip biomarker system (Bio-Rad, Japan), a platform for SELDI-TOF-MS, and protein profiles were obtained and compared. The cation exchange chip (CM10) and the anion exchange chip (Q10) were used for processing before TOF-MS.**

***Results.*** **nine proteins were significantly overexpressed ($P < 0.05$, Student t-test) in patients with gastric cancer compared to patients with gastritis. Among them, four protein masses with 2929 m/z, 3293 m/z, 3371 m/z, and 4213 m/z were found to be differentially expressed solely in patients suffering from peritoneal dissemination. All peaks were processed on CM10 chips. Employing one data mining method, CART (classification and regression trees), gastric cancer patients with peritoneal dissemination were successfully separated from those who had no peritoneal seeding.**

***Conclusion.*** **A validation study with a larger number of samples is mandatory; however, the detected peaks here might be candidates for biomarkers of peritoneal dissemination and/or gastric cancer. Moreover, further analysis of these four proteins might be helpful in revealing the mechanism of peritoneal dissemination, which at present has no cure.** 

***Key Words:*** **gastric cancer; peritoneal dissemination; SELDI-TOF-MS; biomarker; CART.**

## INTRODUCTION

Gastric cancer is the most common malignancy of the gastrointestinal tract among the Japanese and Far Eastern populations and the second most common cancer worldwide [1]. The outcome of patients with advanced or metastatic cancer remains poor, even after curative resection [2].

Peritoneal carcinomatosis or peritoneal dissemination is a common type of metastasis for which surgery is not only futile but often proves to be harmful. However, it is impossible to detect peritoneal metastasis through conventional imaging studies unless the disease has progressed sufficiently to cause obstruction of the intestinal or urinary tract. Staging laparoscopy is therefore required to minimize the possibility of unnecessary laparotomy, while chemotherapy for patients with this pattern of disease is often retarded due to inevitable delay in the detection.

Recently, several authors reported on the Protein Chip approach to analyze serum mass spectra from

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gastric cancer patients by using surface enhanced laser desorption/ionization-time of flight-mass spectrometry (SELDI-TOF-MS) to identify proteins that are differentially expressed between the serum samples of patients with gastric cancer and those from healthy controls [3–5]. In the current study, serum of patients suffering from gastric cancer of various clinical stages was analyzed by the Protein Chip approach. We found some specific proteins that were differentially expressed only in gastric cancer patients with peritoneal dissemination.

## MATERIALS AND METHODS

### Patients and Samples

Serum samples from patients with gastric cancer or gastritis were collected in our hospital from January 2005 to December 2006. All samples were obtained with written patient consent and institutional review board approval. The serum samples were stored as aliquots at –80°C until use.

The control specimens with gastritis ($n = 19$) were collected before gastrointestinal endoscopy. Specimens with gastric cancers ($n = 18$) were collected at the time of gastrectomy. Stages of the cancers ranged from Stage I ($n = 6$) and Stage II or III ($n = 6$) to stage IV ($n = 6$). All Stage IV patients had peritoneal seeding at the time of surgery and underwent exploratory laparotomy only (tumor was not removed). All 18 gastric cancer samples analyzed here had been classified as poorly differentiated adenocarcinoma. The demographic information for all samples is provided in Table 1.

### Protein Chip Array Preparation and SELDI Spectrum Generation

The Protein Chip biomarker system (Bio-Rad, Japan), a platform for SELDI-TOF-MS, was used for protein profile analysis. Serum samples were thawed once before analysis. The cation exchange chip (CM10) and the anion exchange chip (Q10) were used for serum protein SELDI analysis. Serum samples were diluted at a final ratio of 1/10 by PBS, and further diluted by binding/washing buffer for each chip (CM10:50 mM sodium acetate, pH 4, Q10:50 mM Tris-HCl, pH 9, at a final ratio of 1/100). All samples were measured in duplicate and the average data were used for analysis. 20 $\mu$L of diluted serum were put on a spot in the chip for 60 min in humid conditions. Then, each spot was washed with binding/washing buffer three times for 5 min and rinsed with 20 $\mu$L of MilliQ (Millipore, Billerica, MA) twice and then dried at room temperature. Within 30 min, 0.5 $\mu$L of saturated energy absorbing molecule (EAM) solution (sinapinic acid in 50% acetonitrile and 0.5% trifluoroacetic acid) was applied to each spot. These spots were dried and then subjected to another application of saturated EAM solution. After air drying, mass spectrometry was carried out with a chip reader (PCS4000; Bio-Rad), which uses an automated data collection protocol with the manufacturer's software (CiphergenExpress Data Manager 3.0). Laser intensity was determined as 4000–10,000 $\mu$J and focus mass as 4000 Da and 10,000 Da on the basis of the maximum protein peak yield and of reproducibility. After two warming shots, the sum of the data by 10 laser shots with decided laser intensity and 53 points in one spot were collected. External calibration of the instrument was carried out using the manufacturer's standard (All-in-1 Peptide molecular weight standard; Bio-Rad).

### Peak Detection of SELDI Spectra

Peak detection was carried out with the same Protein Chip software. All of the spectra were complied and normalized to the total ion current of an $m/z$ value more than 1500, and the baselines were subtracted. The part of the spectrum with $m/z$ values less than 2000 was not used for analysis because signals from EAM generally interfered with peak detection in this area. Peaks with $m/z$ values were auto-detected when the signal-to-noise ratio was greater than 2.0.

### Statistics

To identify the proteins that discriminate gastric cancer patients from those with gastritis, we compared the protein profiling. For various peaks, $P$ values were calculated on the basis of Student's $t$-test (CiphergenExpress Data Manager 3.0) and a $P$ value of less than 0.05 was considered as statistically significant. At the same time, AUC (area under the ROC curve) of each peak is calculated.

Furthermore, gastric cancer patients were classified histologically (TNM classification) and peaks unique to peritoneal seeding were investigated. Here, a Mann-Whitney test (Wilcoxon test) was applied for comparing small data sets. Finally, data mining of CART (classification and regression trees) was performed as described previously [3]. A decision tree was generated by using the Gini method with nonlinear combination, employing open software R [6]. The complexity parameter was set to 0.01.

## RESULTS

### Detection of Proteins That Are Elevated in Gastric Cancer

Figure 1 is an example of a representative protein spectrum obtained after CM10 processing and SELDI-MS that shows the protein masses. In our experiments, using CM10 and Q10 chips, approximately 500 proteins

**TABLE 1**

**General Information for the Analyzed Patients**

| | Histological classification | | | |
|---|---|---|---|---|
| | Stage I | Stages II or III | Stage IV | Gastritis |
| Sample number | 6 | 6 | 6 | 19 |
| Male/female | 2/4 | 3/3 | 4/2 | 5/14 |
| Age (mean) | 48-88 (63.8) | 48-88 (71.2) | 42-72 (50.3) | 42-88 (60.7) |
| *H. pyrori* status | NT | NT | NT | 11 Positive |
| Liver metastasis | 0 | 0 | 0 | – |
| Peritoneal seeding | 0 | 0 | 6 | – |

*Cancer is all poorly differentiated adenocarcinoma.