Surface-enhanced laser desorption ionization time-of-flight mass spectrometry used to screen serum diagnostic markers of colon cancer recurrence *in situ* following surgery

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Abstract. The aim of the present study was to identify specific serum biomarkers in patients with colon cancer recurrence *in situ* following surgery. The study was conducted at the Renmin Hospital of Wuhan University (Wuhan, China) between January 2012 and January 2014. Surface-enhanced laser desorption ionization time-of-flight mass spectrometry was used to compare and analyze the serum protein profiles of patients with (n=50) and patients without (n=50) recurrence *in situ*. Biomarker Wizard software was used to analyze and obtain the protein spectrum. In total, nine protein peaks demonstrated statistically significant differences between the recurrence and non-recurrence group (P<0.05), which included two protein peaks (7,731.3 Da and 8,266.5 Da). The two protein peaks were highly expressed in patients with colon cancer recurrence *in situ* following surgery, but lowly expressed in patients without recurrence. Therefore, the two protein peaks may represent potential biomarkers for the prediction of colon cancer recurrence *in situ* following surgical treatment.

Introduction

Colon cancer is a tumor of the large intestine, which accounts for >9% of all the cancer cases (1), and is the third most common cancer worldwide (2). Surgery is the standard treatment strategy for patients in the early stages of colon cancer. However, recurrence and metastasis often occur following surgery. Therefore, identifying the predictors of early recurrence and metastasis in patients with colon cancer is important (3). Currently, proteomics is at the frontier of life sciences. Protein fingerprinting, which is one of the technologies used to study the proteome, includes surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) and protein chip techniques. SELDI-TOF-MS is a novel and comparative technology used in proteomics (4-6). The application of comparative technology for proteomics, which screens for markers of tumorigenesis, tumor development and metastasis, is extremely promising (7). Our previous studies have found that certain protein peaks may represent specific biomarkers for colorectal adenomas usign SELDI-TOF-MS technology (8). However, data regarding how to predict early tumor recurrence *in situ* following colon cancer surgery is limited. The present study used SELDI-TOF-MS technology to analyze differences in the serum protein fingerprints of patients with and without tumor recurrence *in situ* following colon cancer surgery. In addition, specific proteins were screened, with the aim of identifying potential biomarkers of tumor recurrence *in situ*.

Materials and methods

Participants. In total, 50 patients with colon cancer recurrence and 50 patients without colon cancer recurrence were selected between January 2012 and January 2014 at the Department of Gastroenterology, Renmin Hospital of Wuhan University (Wuhan, China). The group presenting *in situ* colon cancer recurrence following surgery included patients with anastomotic recurrence of a malignant tumor within five years after the radical resection of a colon tumor. By contrast, the group without colon cancer recurrence following surgery included patients without cancer recurrence within five years after surgery. Overall, 52 patients were male and 48 were female. The mean age was 66.1±5.5 years. The clinical presentation of the patients was confirmed by colonoscopy and histopathological biopsies. The patients did not suffer from any other diseases that affect the serum protein content, including liver or kidney disease, or an endocrine disorder. All the participants were of Chinese ethnicity. The study was approved by the Ethics Committee of Renmin Hospital of Wuhan University. All the patients accepted and signed informed consent.

Patient samples and protein profiling. Following fasting, 5 ml of venous blood was obtained from each of the 100 patients from the recurrence and non-recurrence groups. The samples

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Table I. Differences in protein expression levels following serum protein fingerprinting in the colon cancer recurrence and non-recurrence groups.

		Average intensity of protein peak (mean ± SD)	
Protein peak, Da	P-value	Recurrence group	Non-recurrence group
2,687.5	0.0300	19.63±4.32	14.09±3.34
4,506.1	0.0400	14.23±5.16	10.55±4.76
5,571.2	0.0300	1.37±0.33	0.96±0.29
6,879.3	0.0200	10.02±3.18	7.11±3.02
7,731.3	0.0002	17.26±5.39	3.66±1.12
8,266.5	0.0003	12.97±3.77	3.56±1.11
8,969.7	0.0300	10.50±3.56	15.12±5.78
12,408.1	0.0300	3.76±1.09	1.39±0.27
18,257.6	0.0300	7.22±2.22	4.42±1.57

SD, standard deviation.

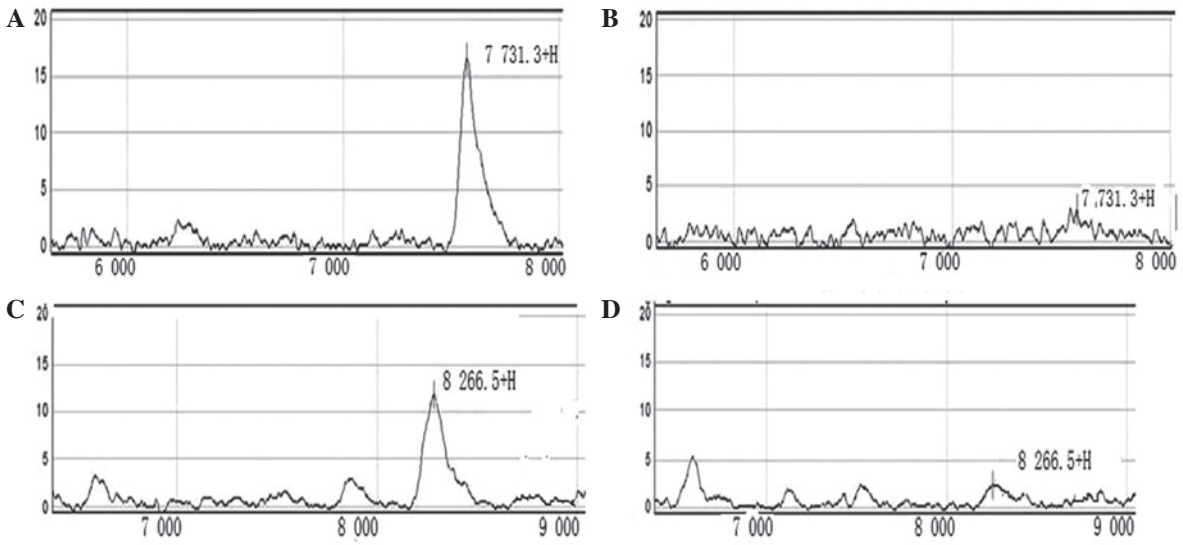

Figure 1. Expression of the 7,731.3 Da protein in (A) the recurrence and (B) non-recurrence groups. Expression of the 8,266.5 Da protein in (C) the recurrence and (D) non-recurrence groups.

were then placed in drying tubes and centrifuged at 2,504 x g for 15 min at 4˚C. Next, the upper serum was collected and transferred into Eppendorf tubes (Sigma-Aldrich, St. Louis, MO, USA) which were placed in a -80˚C refrigerator and reserved.

The serum samples were then removed from the -80˚C refrigerator, thawed and centrifuged at 626 x g for 10 min at 4˚C. Next, 10 μl was collected from each sample and placed in a 1.5-ml centrifuge tube with 20 μl U9 buffer (Ciphergen Biosystems, Inc., Fremont, CA, USA). The tubes were then centrifuged at 626 x g for 30 min at 4˚C, which lead to protein denaturation.

Subsequently, 100 μl WCX2 beads (Ciphergen Biosystems, Inc.) were added into a 200-μl Eppendorf tube and fixed onto a magnetic rack. The supernatant was then discarded after 1.5 min. In total, 100 μl WCX buffer solution (Ciphergen Biosystems, Inc.) was added for 5 min in order to pre-activate the beads. Next, 10 μl processed serum sample was added to the activated beads, mixed well and incubated at room temperature for 30 min. The samples were then set onto the magnetic rack, and the supernatant was discarded after 1.5 min. In total, 100 μl WCX buffer solution was added to eluate the WCX2 beads. Eluant (10 μL 1% trifluoroacetic acid) was added and 1 μl eluent that was rich in proteins was loaded onto an Au/steel chip (Ciphergen Biosystems, Inc.) along with 1 μl sinapic acid saturated solution (Sigma-Aldrich), air-dried and placed into a chip reader for analysis (8).

Using a PBS Type II protein chip reader (Ciphergen Biosystems, Inc.) to analyze the Au/steel chip, the computer converted the raw data into protein fingerprinting at a speed of $1x10^9$/sec. Ciphergen protein chip 3.0 software (Ciphergen Biosystems, Inc., Fremont, CA, USA) was used to calibrate the data. The abscissa of the mass spectrum represents the mass to charge ratio (M/Z) of proteins, whilst the ordinate represents the relative value.

Statistical analysis. Statistical analysis was performed using Biomarker Wizard version 3.1.0 software (Ciphergen Biosystems, Inc.). In addition, the data were processed using the SPSS software for Windows (version 17.0; SPSS Inc., Chicago, IL, USA). Differences in data between the two groups were analyzed using the t-test, while the χ^2 test was used to quantify the data. P<0.05 was considered to indicate a statistically significant difference.

Results

Comparison of age and gender. In the recurrence (mean age, 67.3±4.5 years; male, 28; female, 22) and non-recurrence groups (mean age, 65.9±5.7 years; male, 24; female, 26), the differences in the mean age and gender were not found to be statistically significant (P>0.05).

Screening the differences of serum protein peaks from patients with and without colon cancer recurrence in situ following surgery. The protein molecular masses were set at 2,000-20,000 Da. A protein peak of <2,000 Da was automatically cleared in order to exclude the impact of certain chemical compositions, such as sinapic acid, sodium acetate and trifluoroacetate, on the experimental results. Using the Biomarker Wizard software, nine protein peaks were identified, which exhibited statistically significant differences between the recurrence and non-recurrence groups (P<0.05), including the 2,687.5, 4,506.1, 5,571.2, 6,879.3, 7,731.3, 8,266.5, 8,969.7, 12,408.1 and 18,257.6 Da proteins. Upon comparing the nine protein peaks, two protein peaks, 7,731.3 Da and 8,266.5 Da, were found to have a value of P<0.001 (Table I; Fig. 1).

Discussion

Each year ~930,000 new cases of colorectal cancer are diagnosed worldwide (9). With regard to digestive tract tumors, the incidence of colorectal cancer is next only to gastric cancer. Colorectal cancer has become one of the three major types of cancer in China, with its incidence growing at a rate of 4.2% per year, far exceeding that of the international average (10). The principle of colon cancer treatment involves surgical excision combined with post-surgical chemotherapy and radiotherapy to reduce recurrence and metastasis, as well as to improve survival. However, a number of colon cancer patients experience *in situ* recurrence at different times following surgery, resulting in significant reductions in survival rates (11). Identifying an early index for *in situ* recurrence may aid with early treatment and, therefore, prolong survival. Therefore, the development of a simple, quick and minimally invasive method to accurately detect early recurrence following colon cancer surgery is essential.

In recent years, proteomic technology has developed rapidly. Proteomic technology is considered to be a potentially powerful mean of identifying the mechanisms involved in cancer and provide effective control measures (12). SELDI-TOF-MS is widely used for the early diagnosis of cancer and the early prediction of coronary heart disease and other disorders (8,13,14). It is able to detect the small proteins that are closely associated with tumorigenesis. A previous study indicated that, at present, SELDI-TOF-MS is the most promising method for the early detection of tumors (15).

The present study used SELDI-TOF-MS to compare and analyze the serum protein fingerprints of patients with (n=50) or without (n=50) colon cancer recurrence *in situ* following surgery. The Biomarker Wizard software was used to conduct statistical analysis. The results demonstrated the presence of nine protein peaks that exhibited statistically significant differences between the two study groups (P<0.05). In particular, two protein peaks, corresponding to 7,731.3 Da and 8,266.5 Da, exhibited a value of P<0.001. The identification of the two protein peaks is important for predicting the likelihood of recurrence *in situ* of colon cancer following surgery. In addition, these peaks provide information regarding the early prediction of post-operative recurrence and may assist the identification of specific biomarkers for the prediction of *in situ* recurrence following colon cancer surgery.

In order to conduct SELDI-TOF-MS, only a blood sample is required; therefore, it is a relatively simple technique compared with other clinical examinations (16). Furthermore, SELDI-TOF-MS is able to detect highly-specific serum proteins in patients with colon cancer recurrence *in situ* following surgery. Using this technology as an early diagnostic method has broad prospects. SELDI-TOF-MS is therefore believed to be an effective method for the early detection and diagnosis of colon cancer recurrence following surgery.

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