Analysis of variabilities of serum proteomic spectra in patients with gastric cancer before and after operation

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AIM: To study the variabilities of serum proteomic spectra in patients with gastric cancer before and after operation in order to detect the specific protein markers that can be used for quick diagnosis of gastric cancer.

METHODS: Proteomic spectra of 46 serum samples from patients with gastric cancer before and after operation and 40 from normal individuals were generated by IMAC-Cu protein chip and surface-enhanced laser desorption/ionization time of flight mass spectrometry.

RESULTS: Fourteen differentially expressed proteins in serum were screened by analysis of proteomic spectra of preoperative patients and normal individuals. We obtained 4 proteins (heat shock protein 27, glucose-regulated protein, prohibitin, protein disulfide isomerase A3) making up marker pattern which was able to class the patient-team and normal-team. These marker patterns yielded 95.7% sensitivity and 92.5% specificity, respectively. The proteins over-expressed in serum of preoperative patients were obviously down-regulated.

CONCLUSION: Specific protein markers of gastric cancer can be used for the quick diagnosis of gastric cancer and judgment of prognosis. SELDI-TOF-MS is a useful tool for the detection and identification of new protein markers in serum.

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Key words: Gastric cancer; Proteome; Surface-enhanced laser desorption/ionization time of flight mass spectrometry protein chip technology; Specific marker

INTRODUCTION

Gastric cancer is a common fast growing malignant tumor and takes the first place of the malignant tumors of digestive canal. About 160,000 deaths of gastric cancer patients occur every year in China[1]. It threatens people’s health in our country. The clinical diagnostic methods of gastric cancer are usually physical and pathologic diagnosis, which can only generally find the patients with intermediate stage. The technologies are complex and the patients wait a final diagnosis for a long time. We can only detect the specific protein markers expressed in gastric cancer by biochemical method to achieve early, quick diagnosis. But so far, no marker is completely specific to gastric cancer. Since the sensitivity and specificity of clinical diagnostic method of gastric cancer are not high, it is urgent to explore and set up an early gastric cancer diagnostic method which is easier, quicker and has a higher sensitivity and specificity.

We utilized surface-enhanced laser desorption/ionization time of flight mass spectrometry (SELDI-TOF-MS) protein chip technology to contrast and analyze the changes of serum proteomic spectra in patients with gastric cancer before and after operation in order to screen the specific protein markers that could be used for diagnosing gastric cancer and judgment of prognosis.

MATERIALS AND METHODS

General documents

We chose 46 patients with gastric cancer from Department of Oncosurgery of our hospital in 2003 and 2004. Of the 46 patients, 5 were early cases, accounting for 10.9% of the total, and 41 were in intermediate and advanced stage, accounting for 89.1% of the total. The proportion of men and women was 2.2:1.0, the average age was 51 years. Forty healthy people served as the control group, the proportion of men and women was 2.1:1.0, their average age was 49 years. Before and after 15d of operation 5mL axenic venous blood was drawn from each patient on an empty stomach in the early morning and stored at -80 °C. All the samples were provided by oncosurgery Department...
in our hospital. There was not statistical difference in sex proportion and age between the patient and control groups, and there was no relevant disease that could change serum protein content in the two groups.

**Materials**

Urea, acetonitrile, cibacron blue, 3-glycethylamine-1-propanesulfonic acid (CHAPS), trifluoroacetic acid (TFA), sinapinic acid (SPA) were all bought from Sigma Company. SELDI-TOF-MS, K30 size selection spin column and IMAC3 protein chip were produced by Ciphergen Company in USA.

**Serum sample preparation**

The samples stored at -80 °C were deliquesced at common temperature, cibacron blue and K30 size selection spin column were used to remove albumin in the samples. Then we used IMAC buffer solution (100 mmol/L NaPO4 +250 mmol/L NaCl, pH 7.0) to dilute 20 μL serum sample.

**Preparation of IMAC3 protein chip**

We installed the IMAC#3 chip onto the bio-processor, added 50 μL of copper sulphate (100 mmol/L) into every hole, spun it at 4 °C with the rotation speed at 200 per minute for 5 min, made copper ion with active surface of the chip. The remaining blotted copper sulphate was used to flush the chip in deionized water. Fifty μL of acetic acid sodium (100 mmol/L, pH 4.0) was added and spun to etute un-conjugated copper ion. The liquid was discarded and 150 μL balanced solution (50 mmol/L HEPES, pH 7.0) was added into every hole. Then 50 μL sample was added and incubated at 4 °C for 1 h. Excess liquid was discarded and 150 μL HEPES balanced solution flushing 3 times was used to etute the protein or peptide pieces that did not combine with the surface of the chip. Then we used PAP hydrophobic pen to draw the sample hole surrounding the chip and air dried it. After that, we added 0.5 μL SPA solution twice in every hole.

**Data reading and result analysis**

The prepared chip was analyzed by the analytic equipment of protein chip. Data were obtained by using Ciphergen Proteinchip software. Before the data were obtained, angiotensin peptide was used to correct the equipment to make the mass deviation of system less than 0.1%. The parameters of the data reading were as follows. Laser intensity was 175, detection sensibility was 8, grading-up molecular weight limit was 1500-100000 Da, best focal setting centre was 6000, parameter limit of data collection was 20-80. Biomarker Wizard software was used to process the obtained data, the proteinic wave crest intensity was calculated, which had the same e/m. Different degrees were indicated by P value.

**Statistical analysis**

Data were analyzed using Biomarker Wizard software and Biomarker Patten software. P<0.05 was considered statistically significant.

**RESULTS**

**Distributed results of serum proteomic spectra between patients with gastric cancer before operation and normal control**

**Peak detection**

The protein molecular weight limit detected in the samples from 46 patients with gastric cancer before operation and 40 healthy people was set between 1500 and 100000 Da. The proteins or peptide pieces less than 1500 Da were precluded, because between 0 and 1500 Da there were many agent compositions, SPA and other chemical compositions which could influence data analysis. The IMAC#3 array was analyzed by the analytic equipment of protein chip. The proteins obtained were expressed as spectrum and 54126 wave crests were obtained.

**Peak alignment and proteomic spectrum analysis**

Spectra obtained were analyzed by using Biomarker Wizard software. The content of most proteins in samples was generally identical in the preoperative and control groups, but 33 proteins were still differentially expressed in the two groups, 14 proteins of which were significantly differentially expressed in the 33 proteins in the two groups (P<0.05). In these 14 proteins, seven proteins were over-expressed in the 46 patients with gastric cancer before operation, the average molecular weight of them was 22.33, 22.59, 28.42, 29.90, 35.72, 42.46 and 56.77 kDa (named gastric cancer over-expressed serum proteins 1-7), respectively. Compared with control group, seven proteins were under-expressed, the average molecular weights of them were 17.16, 22.02, 30.77, 46.73, 53.40, 77.07 and 80.02 kDa (named gastric cancer under-expressed serum proteins 1-7), respectively. Fourteen proteins were identified, the result showed that the seven over-expressed proteins were heat shock protein (hsp) 27, transgelin, glucose-regulated protein, prohibitin, NSP3, unnamed protein product and protein disulfide isomerase A3 (Table 1). The seven under-expressed proteins were p20, nucleoside diphosphate isomerase A, apolipoprotein A-1, alpha 1 antitrypsin, desmin, serotransferrin and serum albumin (Table 2).

**Screening for gastric cancer serum protein markers and establishment of diagnostic pattern**

The data of the differentially expressed proteins were used to establish a database. Using Biomarker Pattern intellligent statistical analysis software to select corresponding conditions, we analyzed the data obtained from preoperative and control groups and obtained the specific proteinic markers of gastric cancer. The results showed that using the pattern

| Table 1 Seven over-expressed proteins in gastric cancer |
|------------------|------------------|------------------|
| **Protein No.** | **Average molecular weight (kDa)** | **Protein identified** |
| 1               | 22.33            | Heat shock protein 27 |
| 2               | 22.59            | Transgelin         |
| 3               | 28.42            | Glucose-regulated protein |
| 4               | 29.90            | Prohibitin         |
| 5               | 35.72            | NSP3              |
| 6               | 42.46            | Unnamed protein product |
| 7               | 56.77            | Protein disulfide isomerase A3 |
composed of heat shock protein 27, glucose-regulated protein, prohibitin, protein disulfide isomerase A3 could exactly distinguish gastric cancer patients from healthy people. Using these markers, we established the taxonomic tree pattern of gastric cancer diagnosis, which has two layers and three nodes and could exactly distinguish 95.7% of gastric cancer patients from 92.5% healthy people. The study results showed that these marker patterns yielded 95.7% sensitivity and 92.5% specificity, respectively.

**Variabilities of serum proteomic spectra in patients with gastric cancer before and after operation**

The sera of patients with gastric cancer after operation were analyzed by using IMAC#3 protein chip and Biomarker Wizard software with the same condition and parameters. The results were compared with those in the preoperative and control groups. The results showed that the expression of heat shock protein 27, glucose-regulated protein, prohibitin, unnamed protein product and protein disulfide isomerase A3 (namely: gastric cancer over-expressed serum proteins 1, 3, 4, 6, 7) decreased obviously in postoperative group compared with control group. The five proteins had significantly different expression. When compared with the preoperative group, the five proteins had significantly different expression ($P<0.05$). NSP3 and transgelin (namely: gastric cancer over-expressed serum proteins 2, 5) were not obviously down-regulated after operation and still over-expressed. When compared with the preoperative group, they were not significantly expressed. When compared with control group, they were significantly expressed ($P<0.05$) (Table 3). Compared with the preoperative and control groups, the results of serum proteomic spectra in postoperative group showed that serum proteomic spectra had some variabilities and the expression of some proteins with heavier molecular weight was different from that in the above two groups.

**DISCUSSION**

Gastric cancer is a frequently encountered malignant tumor in clinic. Gastric cancer possesses a natural bacterial drug resistance to many chemotherapeutics. The current situations of gastric cancer in our country are high case fatality, low early diagnosis rate, low exaeresis rate and low five-year survival rate. The method to diagnose gastric cancer is very complex. Pathogenesis of gastric cancer is a long process. Multiple factors determine genetic predisposition, which refers to bacterial infection, abnormal changes of many oncogenes and anti- oncogenes, including activation of oncogene, inactivation or absence of anti-oncogene, genetic mutation, etc. It was reported that infection with helicobacter pylori could result in gastric epithelial hyperplasia and apoptosis. Decrease of folacin in cells reduces the methylated level of some DNA fragments, including certain oncogenes. Some scholars believe that the lower the methylated level of genes is the poorer the differentiation of the cancer cell. The deletion of nm23 gene may relate with the transfer process of gastric cancer. It is necessary to find a new, effective and quick diagnostic technique which has a high sensibility and specificity to diagnose gastric cancer.

Before pathological change is discovered, the component and quantity of intracellular proteins may have corresponding changes in the disease. Through dynamic observation of proteins, pristine disease signs can be discovered. Proteomic technology has made the screening possible and can establish a new molecular diagnostic technology for clinical disease diagnosis, especially for tumor diagnosis. Proteomic studies could lead to molecular characterization of cellular events associated with cancer progression, signaling and developmental stages. Cancer-specific protein markers are the basis for developing new methods for early diagnosis of gastric cancer and its progression. Genes work at protein level. Since the proteomic results can express both the intrinsic genetic effect on cells and the impact of its environment, it is very valuable to determine biomarkers of tumor. In the past, the main technologies used in protein research are 2D electrophoresis (2DE), high performance liquid chromatography (HPLC), mass spectrum, etc. Because information obtained is not enough, the development of proteomics is confined. SELDI-TOF-MS protein chip technology is a new proteomic technology and can be used in high-flux analysis. Because the protein chip system based on this technology is widely used to screen early diagnostic markers of tumor, significant results have been achieved. Eggeling et al. have obtained the proteomic spectral variation of renal cancer by using this technology. Paweletz et al. investigated the proteomic spectral variation of liquid drawn from nipple and showed that...

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**Table 2 Seven under-expressed proteins in gastric cancer**

| Protein No. | Average molecular weight (kDa) | Protein identified |
|-------------|--------------------------------|--------------------|
| 1           | 17.16                          | P20                |
| 2           | 22.02                          | Nucleoside diphosphate isomerase A |
| 3           | 30.77                          | Apolipoprotein A-1  |
| 4           | 46.73                          | Alpha 1 antitrypsin |
| 5           | 53.40                          | Desmin             |
| 6           | 70.07                          | Serotransferrin     |
| 7           | 80.02                          | Serum albumin      |

**Table 3 Variabilities of gastric cancer serum over-expressed proteins before and after operation**

| Proteins                     | Molecular weight (kDa) | Expression preoperation | Expression postoperation |
|------------------------------|------------------------|-------------------------|-------------------------|
| Heat shock protein 27        | 22.33                  | Over-expressed$^a$      | Normal$^a$              |
| Transgelin                   | 22.59                  | Over-expressed$^a$      | Over-expressed$^a$      |
| Glucose-regulated protein    | 28.42                  | Over-expressed$^a$      | Normal$^a$              |
| Prohibitin                   | 29.90                  | Over-expressed$^a$      | Normal$^a$              |
| NSP3                         | 35.72                  | Over-expressed$^a$      | Over-expressed$^a$      |
| Unnamed protein product      | 42.46                  | Over-expressed$^a$      | Normal$^a$              |
| Protein disulfide isomerase A3| 56.77                  | Over-expressed$^a$      | Normal$^a$              |

$^aP<0.05$ vs control group; $^\prime P<0.05$ vs preoperative group.
the proteomic spectra in patients with mammary cancer are significantly different from those in healthy people. Vlahou et al. have screened out five specific markers of bladder cancer from urine of patients with bladder cancer. These findings indicate that SELDI-TOF-MS protein chip technology is one of the main technologies of proteomics, especially suitable for screening the specific markers of tumor.

The results of our study showed that 33 proteins were differentially expressed in preoperative and control groups, 14 proteins of which were significantly expressed (P<0.05). In these 14 proteins, seven proteins were over-expressed and seven proteins were under-expressed. Through the identification of the 14 proteins, the seven over-expressed proteins were heat shock protein 27, transgelin, glucose-regulated protein, prohibitin, NSP3, unnamed protein product and product disulfide isomerase A3, while the seven under-expressed proteins were p20, nucleoside diphosphate isomerase A, apolipoprotein A-1, alpha 1 antitrypsin, desmin, serotransferrin and serum albumin. The data of differentially expressed proteins were used to establish the database. The Biomarker Pattern intellective statistical analysis software was used to analyze the data in the database. We established a diagnostic pattern composed of heat shock protein 27, glucose-regulated protein, prohibitin, protein disulfide isomerase A3, which can exactly distinguish gastric cancer patients from healthy people. If these markers are used to diagnose early gastric cancer, they are able to yield a 95.7% sensitivity and a 92.5% specificity, respectively.

The serum proteomic spectra also changed in patients with gastric cancer after operation. The five formerly over-expressed proteins, namely heat shock protein 27, glucose-regulated protein, prohibitin, unnamed protein product and protein disulfide isomerase A3, were obviously down-regulated. When compared with control group, the five proteins were not significantly expressed (P>0.05), but the two proteins, namely transgelin and NSP3, were not obviously down-regulated. When compared with control group, the two proteins were over-expressed (P<0.05). The results of our study suggest that using the diagnostic pattern composed of the above four proteins to diagnose gastric cancer, can obtain a high sensitivity and specificity and it can be widely used for clinical diagnosis of gastric cancer. But because the cases in early stage accounted for a small proportion of the samples in our research, the technology can only be used to diagnose gastric cancer. Before we give a conclusion whether the technology can be used to screen early gastric cancer, we must collect more cases of early gastric cancer.

In a word, using the diagnostic pattern composed of heat shock protein 27, glucose-regulated protein, prohibitin, protein disulfide isomerase A3 to diagnose gastric cancer can obtain higher positive rate, higher sensitivity and specificity. It can be used to diagnose gastric cancer quickly and exactly.

In conclusion, SELDI-TOF-MS protein chip technology has lots of advantages and is a new effective technology for study of proteins.

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