RESEARCH COMMUNICATION

Detection and Prognostic Analysis of Serum Protein Expression in Esophageal Squamous Cell Cancer

Hong Jiang[1], Xiao-Hong Wang[2]*, Xin-Min Yu[2], Zhi-Guo Zheng[3]

Abstract

Objective: To assess differences in serum proteins in esophageal squamous cell carcinoma patients. Methods: 144 esophageal squamous cell carcinoma patients and 50 healthy volunteers were included in this study, with surface-enhanced laser desorption-ionization time-of-flight mass spectrometry and weak cation exchange magnetic beads. Follow-up allowed the relations between serum proteins and prognosis to be analyzed. Results: A total of 93 protein peaks were detected (molecular weight range: 1500-30000), 10 demonstrating statistically significant differences. There were no differences in protein peaks between 92 patients with a survival more than 2 years and 52 patients with survival less than 2 years. There were two significantly different protein peaks between 45 stage II patients with a survival more than 2 years and 14 stage II patients with survival less than 2 years. There was one significantly different protein peak between 22 stage III patients with a survival more than 2 years and 29 stage III patients with survival less than 2 years. Conclusion: Differences of serum proteins in esophageal squamous cell carcinoma are related to prognosis of patients. The protein fingerprint can be helpful for clinical diagnosis and treatment.

Keywords: Esophageal cancer - prognosis - serum protein fingerprint

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Introduction

Esophageal cancer is a common clinical malignancy. According to the Cancer Statistic Report from the International Agency for Research on Cancer (IARC), there were 462,000 new cases in 2002 and 386,000 deaths. Esophageal cancer is the 8th most common malignancy worldwide and one of the six most lethal diseases. The treatment of esophageal cancer is not effective enough, because the majority of patients are diagnosed too late. Specific markers will contribute to an early diagnosis and predict patients' prognosis. Therefore, to seek efficient molecular markers is valuable for diagnosis and treatment of esophageal cancer.

Recently, the rapid development of proteomics promotes the studies on tumor markers. During the last decade, surface-enhanced laser desorption-ionization time-of-flight mass spectrometry (SELDI-TOF-MS) has been a widespread proteomics implement in oncology study. A diagnostic model for esophageal squamous cell carcinoma and cervical squamous cell carcinoma has been devloped (Xia et al., 2008).

According to the pattern of esophageal squamous cell carcinoma, serum protein changes are related to the occurrence of esophageal cancer. It still remains unclear if protein changes relate to prognosis. In the present study, SELDI-TOF-MS and weak cation exchange magnetic bead were used to examine the changes of serum protein expression in newly diagnosed patients with esophageal cancer to further identify esophageal cancer-related proteins. During the follow-up, the relationship between serum protein differences and prognosis was analyzed in esophageal cancer.

Materials and Methods

Patients

From August 2007 to June 2010, peripheral blood samples from 144 newly diagnosed cases with esophageal squamous cell carcinoma (age: 43-75, median age: 60) before surgery were collected. The control group included 50 sex- and age-matched healthy volunteers (age: 36-78, median age: 56). Pathological examination confirmed esophageal squamous cell carcinoma after gastroscopy. The exclusion criteria included hepatitis, acute infection, and concurrent tumors. All patients underwent surgical treatment. Surgical approach was upper abdomenl-right chest or upper abdomen-right chest-left neck. Postoperative pathology examination was performed according to UICC 2009 version of the esophageal cancer staging (stage I: 28 cases, stage II: 59 cases, stage III: 51 cases, stage IV: 6 cases).

*[1]Key Laboratory of Thoracic Tumor Diagnosis (Esophagus, Lung) and Treatment Technology Research, Department of Thoracic Surgery, [2]Department of Chemotherapy, Zhejiang Cancer Hospital, [3]Zhejiang Cancer Institute, Hangzhou, China *For correspondence: jhmecn@163.com*

Instruments and Reagents

Acetonitrile, trifluoroacetic acid, SPA (Sinapinic acid), urea, DTT, CHAPS, Tris-HCL, dH2O were purchased from Sigma company. MALDI-TOF-MS (PBSIIc) mass spectrometer was a Ciphergen Biosystems Company product. Weak cation-exchange (WCX) nanobeads, binding buffer, elution were provided by Beijing Saier Di Company. Peripheral venous blood (5 ml) was colleted one day preoperatively.

Sample Collection and Pretreatment

Blood specimens were placed at 4 °C in a refrigerator. One or two hours later, residual cell fragment was removed after centrifugation at 4000 r/min for 5 minutes and 14000 r/min for 5 minutes at 4 °C. On the ice, serum was transferred to a new centrifuge tube then placed at -80 °C. The serum samples melted on the ice before use. Buffer solution (20 ul, 9 M) was added to 1.5 ml well-marked centrifuge tube (9 mol/L Urea, 2% CHAPS, 50 mmol/L Tris-HCl, 1% DTT, pH 9.0). Then 10 μl serums were added into centrifuge tube, and mixed well. The diluted sample was placed at room temperature for 10 minutes. Then 360 μl binding buffer was added and mixed well.

Magnetic Nanobeads Pretreatment, Loading and Elution

Weak cation-exchange (WCX) nanobeads were loaded into PCR tubes separately, placed on the magnetic processor and absorbed liquid, followed by adding 100 μl binding buffer, mixed well and placed for 5 minutes. Then PCR tubes were placed on the magnetic processor and absorbed liquid twice. Diluted serum sample 100 μl was added into every PCR tube loaded with nanobeads, mixed and placed at room temperature for 15 minutes. And then it was placed on the magnetic processor to absorb unbonded samples. Binding buffer 100 μl was added into every tube, mixed and placed for 5 minutes. Then PCR tubes were placed on the magnetic processor to absorb liquid. The procedure was repeated again. Every tube was added 10 ul eluent, mixed and placed 5 minutes, and placed on the magnetic processor. Supernatant 5 μl was transferred into another PCR tube. Saturated solution of SPA was added and mixed well. Sample 1 μl was added to an Au chip and then was tested after the chip was dry. SPA was a saturated solution of sinapinic acid in 50% CAN (acetonitrile) and 0.5% TFA (trifluoroacetic acid).

Chip Test, Data Acquisition and Parameter Set

PBS-C mass spectrometer was used to read the chip information. NP20 chip calibration equipment including All-in-one standard protein was used to make sure the error range of molecular weight was less than 0.1 %. Chip reading instrument was set as follows: laser intensity 185, detection sensitivity 7, range optimization 1500-30000. Samples at each point were recored for 90 times. Ciphergen ProteinChip software 3.2.1 was used to collecte data.

Follow-up

All patients completed telephone follow-up. The follow-up rate was 97.2% with 4 cases lost to follow-up.

Data Analysis

The main process of data analysis included: (1) Biomarker Wizard software was used to promptly calculate P values for differential peaks of proteins with the same mass to charge ratio (m/z) among groups. An effective protein peak was defined as peaks with a signal-to-noise ratio (S/N) > 5 and an occurrence frequency > 90%. In order to avoid any omissions of minor protein peak, peaks with S/N> 2 and molecular weight deviation < 0.3% were alos considered as an effective protein peak. P values for expression differences of proteins with the same m/z ratio among groups were calculated according to chi-square analysis (Excel was used to export molecular weight and relative intensity of differential proteins). SPSS software was used to study the relationship between survival and protein peaks.

Results

After standardization, original serum protein fingerprints from 33 patients with esophageal squamous cell carcinoma and 33 healthy control subjects was analyzed by Biomarker Wizard software. With molecular weight between 1500 and 30000, 93 protein peaks were identified. Ten protein peaks were significantly different between esophageal squamous cell carcinoma and control groups (P <0.001). The 3908 and 3403 differential peaks in esophageal cancer and control group was shown in Figure 1.

ROC curve analysis by using SPSS 12.0 was performed in more participants including 144 esophageal cancer patients and 50 healthy control subjects. The ROC curve analysis results of 10 differential peaks were shown in Table 1. The area under ROC curve (AUC) of differential peaks larger than 0.8 in 2768, 2745, 3403, 6638, 5972, 6440, 3321 and 3978m/z was of promising diagnostic significance.

There was no difference for protein peaks between 92 patients with a survival more than 2 years and 52 patients with survival less than 2 years.

There were two significantly different protein peaks between 45 stage II patients with a survival more than 2

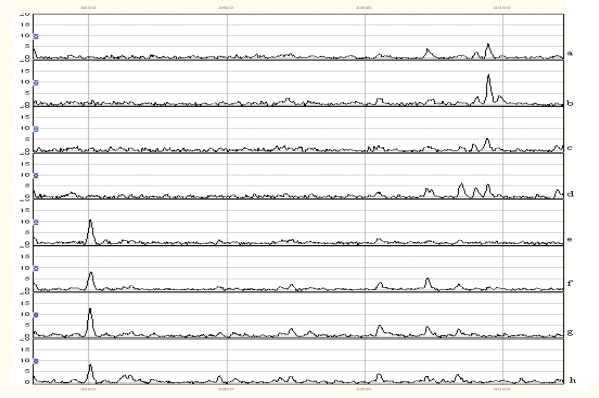

Figure 1. The Original Mass Spectrum of 2 Different Protein Peaks (3978 and 3403) Between Esophageal Cancer Patients and Healthy Controls. Over expression of 3978 m/z in esophageal cancer patients and low expression of 3403 m/z were noted in healthy controls (a-d: esophageal cancer patients; e-h: healthy controls)

Table 1. The Area Under the ROC Curve of 10 Differential Peaksin 144 Esophageal Squamous Cell Carcinoma Patients and 50 Healthy Subjects

Protein (m/z)	ROC Area
2768	0.926
2745	0.944
3403	0.97
6638	0.897
5972	0.812
6440	0.872
4797	0.689
3269	0.678
3321	0.846
3978	0.82

years and 14 stage II patients with survival less than 2 years.

There was one significantly different protein peak between 22 stage III patients with a survival more than 2 years and 29 stage III patients with survival less than 2 years.

There was one significantly different protein peak between 22 stage III patients with a survival more than 2 years and 20 stage III patients with survival 1-2 years.

Discussion

SELDI-TOF-MS was first proposed in 1993. In recent years, this technology has made considerable progress. According to the conclusion drawn from a stage III multicenter clinical trial by U.S. National Cancer Institute (NCI) affiliated early disease detection network, SELDI-TOF-MS is the most promising cancer early detection methods after serum and instrument standardization quality control (Grizzle et al., 2004). Its principle is to use high-energy laser beam to make the analyte in the chip resolved to form ions. On the basis of different mass-to-charge ratio (M/Z), the time of flight of ion in the instrument field is various. As a result, a protein spectrum can be drawn. After computer analysis, data regarding protein molecular weights and concentrations can be recorded.

Its main advantages are listed below: 1) Samples are simple and convenient to access and even directly crude samples. The changes of protein (Panicker et al., 2009; Calvano et al., 2010) were detected in the cervix mucus protein of patients with cervical cancer and urine of prostate cancer patients. 2) Small sample volume and prompt detection. 3) high sensitivity and specificity (Guo et al., 2011). 4) Protein molecular weight range is wide. Low-molecular weight, low-abundance proteins can be found. 5) Widespread in clinical use: tumor etiology, diagnosis, efficacy monitoring of various treatment methods and prognostic evaluation of patients (Kohli et al., 2006; Ren et al., 2009).

But it still has several disadvantages in protein markers and early diagnosis of disease. First, most experiments focus on primary description of differential protein peak and molecular weight. Protein sequence, conformation, purification and features need more attention. Thereby some specific proteins can not be identified from much further way. In addition, the reproducibility of SELDI-TOF-MS needs to be established because of controversial conclusions regarding the same research subject.

Recently, many scholars have succeeded in finding new tumor markers in tumor such as cervical cancer (Piyathilake et al., 2007; Xia et al., 2008), ovarian cancer (Zhang et al., 2006), colorectal cancer (Xu et al., 2006), prostate cancer (Malik et al., 2007; Mclerran et al., 2008), breast cancer (Ricolleau et al., 2006), bladder cancer (Langbein et al., 2006) by SELDI-TOF-MS, and established relavant diagnosis model of tumor protein fingerprint whose sensitivity and specificity are higher than existent tumor markers. Even more promisingly, the high-resolution mass spectrometer in combination with statistical advances had a higher sensitivity and specificity (Yu et al., 2005; Cadron et al., 2009).

At the same time, many researchers studied the changes of serum proteins in the occurrence and development of esophageal cancer and reported various proteins were related to the occurrence and metastasis of esophageal cancer (Breton et al., 2008; Liu et al., 2010). We have established a diagnosis model of esophageal cancer and cervical squamous cell carcinoma among patients of Zhejiang Province. Through the comparison between patients of esophageal cancer or cervical cancer and healthy volunteers, several significant differential protein peaks was identified. However, domestic and international researches on relevance of serum protein changes and prognosis in patients with esophageal cancer are less reported.

In this study, there were no significantly different protein peaks between 92 patients with survival more than 2 years and with 52 patients with survival less than 2 years. This was probably because of different pathological stages ant related interference factors; Therefore, patients of stage II and patients of stage III were compared, respectively. And it was concluded that differences of serum protein spectrum in esophageal squamous cell carcinoma were related with the prognosis.

Two statistically different protein peaks were also found after analysis of serum albumin in patients with lymph node metastasis or not. There were 6 statistically different protein peaks between patients without lymph node metastasis and those with distant metastasis in this study. It implied that there were significantly different gradients of relative content of serum proteins between early and middle-advanced esophageal cancer patients. The lack of expression of certain proteins might lead to tumor metastasis indicating that these proteins probably could be related markers of tumor metastasis. There was no statistically different protein peaks between patients with lymph node metastasis and those with distant metastasis in the present study. The underlying cause might originate from unknown distant mircometastasis in middle-advanced esophageal cancer patients with lymph node metastasis. One liver metastasis and 1 lung metastases were confirmed in this study which had no metastasis evidence preoperatively.. The small cases of distant metastasis (n=2) in this study must be taken into account which might introduce a statistical error.

Nowadays, some scholars have also established

diagnosis model of esophageal cancer protein fingerprint in different races, including esophageal squamous cell carcinoma and adenocarcinoma (Hammoud et al., 2007; Xu et al., 2009; Zhang et al., 2011). Acoording to our research, there were 10 different protein expressions between esophageal cancer patients with a family history and patients without a family history. And these differential proteins were over expressed in esophageal cancer patients with a family history. Whether there are differences of protein expression between patients inform esophageal cancer epidemic areas – Tiantai and Xianju and other places will requie further studies in the future.

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