Clin Oncol Cancer Res (2009) 6: 317-321
DOI 10.1007/s11805-009-0317-2

Discovery of Metastasis-Associated Biomarkers in Ovarian Cancer Using SELDI-TOF: An in Vitro and Clinical Study

Zhiguo Zheng[1]
Yun Gao[1]
Linhui Gu[1]
Hanzhou Mou[1]
Chihong Zhu[1]
Jianqing Zhu[2]
Shenhua Xu[1]

[1] Zhejiang Cancer Research Institute, [2] Department of Gynecology of Zhejiang Cancer Hospital, Hangzhou 310022, Zhejiang Province, China.

Correspondence to: Shenhua Xu
E-mail: shenhuaxu@hotmail.com

This work was supported by grants from the Medical Scientific Research Foundation and Bureau of Health Care for Senior Officials of Zhejiang Province, China (No.2007B026) and the National Natural Science Foundation of China (No.0471819).

Received May 14, 2009; accepted September 15, 2009.

E-mail: 2008cocr@gmail.com
Tel (Fax): 86-22-2352 2919

OBJECTIVE To identify metastasis-related biomarkers in human ovarian cancer cell lines and in serum.
METHODS We isolated total protein from cell lysis solutions and cultured supernatants from 2 human ovarian cancer cell lines and used SELDI-TOF-MS to detect the differential expression of the proteins in the 2 cell lines. The proteomic spectra were generated using weak cation exchange chips. The biomarkers were validated by analyzing serum proteins or peptides in ovarian cancer patients, relapsed ovarian cancer patients, patients with benign ovarian tumors, and healthy people.
RESULTS Four proteins in the culture supernatant from HO-8910PM cells were up-regulated, relative to the culture supernatant of HO-8910 cells. One protein (3,144 Da m/z value) was up-regulated in both the cell lysis solution and in the culture supernatant of HO-8910PM cells. In addition, expression of the 3,144 Da m/z protein differed significantly between serum from the 26 ovarian cancer patients, from the 22 relapsed ovarian patients and from the 37 healthy women ($P < 0.01$). However, there was no difference between patients with benign ovarian tumors and healthy people ($P > 0.5$).
CONCLUSION Ovarian cancer cell lines with high or low metastatic potential have distinct protein profiles. Protein 3,144 Da m/z could be a useful biomarker for diagnosing ovarian cancer metastasis.

KEY WORDS: ovarian cancer, metastasis, SELDI-TOF.

Introduction

Ovarian cancer is one of the most common tumors of the female reproductive system. Unfortunately, the majority of epithelial ovarian cancers remain clinically undetected until progression to late stage disease, and in one study, only 25% of tumors were detected in stage I upon diagnosis[1]. Despite advancements in cytoreductive surgery and combination chemotherapy over the last 2 decades, long-term survival has remained stable[2]. In order to increase long-term survival, molecular therapeutics must be linked to molecular diagnostics in order to develop individualized therapy[3].

The profiling of low-molecular-weight serum proteins might help reflect the pathologic state of organs and aid in the early detection of cancer. Surface-enhanced laser desorption and ionization time-of-flight (SELDI-TOF) mass spectroscopy (MS) can detect proteins in this range, and several serum biomarkers identified by this method have been used to detect early ovarian cancer[4,5]. Our aim was to

identify metastasis-related biomarkers by comparing the differential protein expression profiles of 2 human ovarian cancer cell lines with different metastasis potentials in vitro. We then aimed to validate these biomarkers by analyzing serum proteins or peptides in ovarian cancer patients, relapsed ovarian cancer patients, women with benign ovarian disorders, and healthy women.

Materials and Methods

Chemicals and apparatus

RPMI1640 was obtained from Gibco and the calf serum from Hangzhou Sijiqing Company. Acetonitrile, trifluoroacetic acid, urea, CHAPS, sinapic acid, DL-dithiothreitol, Tris-HCL, HCL, sodium acetate and water were purchased from Sigma-Aldrich. ProteinChip Biology System (PBSIIc) mass spectrograph, weak cation-exchange (WCX2) protein chips were purchased from Ciphergen Biosystems.

Cell lines

The ovarian cancer cell line HO-8910[6] and the highly metastatic ovarian cancer cell line HO-8910PM[7] were provided by the Zhejiang Cancer Institute. HO-8910 and HO-8910PM were cultured in RPMI1640 including 15% calf serum, at 37°C in 5% CO_2. Calf serum was removed after 24 h. Samples were centrifuged at 15,000 rpm at 4°C for 30 min. The culture supernatant was immediately frozen in aliquots of 100 µl at -80°C. The cells were lysed using RIPA lysis buffer and centrifuged at 15,000 rpm at 4°C for 30 min. The supernatant was immediately frozen in aliquots of 100 µl at -80°C.

Cancer patients and the controls

We used a retrospective sample of 120 serum specimens. All samples were collected from May 2006 to May 2007 with the approval of Zhejiang Cancer Hospital. The study was performed after approval from the institutional Human Investigations Committee. The samples were collected from 26 patients with invasive epithelial ovarian cancer in stages III/IV (median age 53 years, range 37-74 years), and 22 patients with relapsed ovarian cancer (median age 48 years, range 26-67 years). All of the cancer patients had serous tumors. The remaining samples were collected from 35 patients with benign ovarian disorders (median age 42.5 years, range 21-71 years) and 37 healthy controls (median age 39 years, range 24-65 years). The malignant or benign diagnosis was confirmed by pathologists.

Serum samples

Blood samples were collected from the patients before treatment and stored at 2°C-8°C for a maximum of 2 h before centrifugation. They were processed according to standardized protocol. After collection, samples were centrifuged at 4,000 rpm for 6 min, and the serum was immediately frozen in aliquots of 100 µl at -80°C. For proteome fractionation, samples were thawed at room temperature for 5-10 min and processed immediately.

SELDI analysis

The weak cation-exchange (WCX2) ProteinChip was selected for our current study. WCX2 ProteinChip is a weak cation exchange array with a carboxylate surface to bind cationic proteins. The negatively charged carboxylate groups on the surface of the WCX2 chip interact with the positive charges exposed on the target proteins. Per protocol, 10 µl of serum were incubated with 20 µl of U9 (9 mol/ L Urea, 2% CHAPS, 50 mmol/ L Tris-HCl pH 9.0, 1% DTT) at room temperature for 30 min on a platform shaker. After the samples were ready, they were incubated at room temperature for 60 min on a platform shaker, and the array was washed twice with 200 µl of binding buffer for 5 min. Before SELDI analysis, 0.5 µl of a saturated EAM solution (sinapinic acid in 50% acetonitrile/0.5% trifluoroacetic acid) was applied to each chip array twice, allowing the array surface to air dry before each chip array was used. Chips were placed on the Protein Biological System II mass spectrometer reader, and time-of-flight spectra were generated by averaging 90 laser shots collected in the positive mode at a laser intensity of 185 and with a detector sensitivity of 8. Mass accuracy was calibrated on the day of measurement using the all-in-one peptide molecular mass standard (Ciphergen Biosystems).

Statistical analysis

Peak detection was performed using Ciphergen ProteinChip software 3.2 (Ciphergen Biosystems). A mass range of 1,000-50,000 Da was selected. Peak detection involved *i*) baseline subtraction, *ii*) mass accuracy calibration, and *iii*) automatic peak detection. Using Biomarker Wizard (Ciphergen Biosystems), biomarkers which were consistent with protein peaks across multiple spectra were identified. After biomarker identification, the quality and intensity of the readings of the selected peaks were manually reconfirmed from raw spectra. The Receiver Operating Characteristic (ROC) curve (AUC) was computed to identify the peaks with the highest potential to discriminate between ovarian cancers and benign tumors. For cell lines, different mass spectrometry peaks were defined as intensity ratios > 2[8].

Protein identification

Proteins were identified using TagIdent (http://expasy.org/tools/tagident.html). The peak sizes were determined by SELDI-TOF-MS analysis. The search criteria allowed for a 0.1% size error.

CA125 assay

CA125 assay was performed by chemiluminescent microparticle immunoassay using the Architect i2000

system (Abbott, USA). The cut-off value was set at 35 U/ml.

Results

Differential protein expression in HO-8910PM and HO-8910 cells

Four distinct protein peaks (3011, 3021, 3144 and 6635 Da) were detected in the culture supernatants of HO-8910PM and HO-8910 cells, all of which were relatively up-regulated in HO-8910PM cells (Table 1). The 3,021 m/z and 3,144 m/z peaks are shown in Fig.1. In addition, 11 distinct protein peaks were detected in the lysis solution from HO-8910PM and HO-8910 cells. A distinct 3,144 m/z peak was found in both the lysis solutions and culture supernatants from HO-8910PM and HO-8910 cells.

Table 1. The average peak intensities of 4 distinct protein spectra found in the culture supernatants of HO-8910PM and HO-8910 cells.

	Average peak intensity of the protein spectra	
Protein (m/z)	HO-8910PM	HO-8910
3,011	3.3	0.8
3,021	4.3	0.6
3,144	10.3	3.7
6,635	5.5	2.5

The different concentrations of 3144m/z detected in sera

The average values of 3,144m/z in ovarian cancer patients in stage III-IV, relapsed ovarian cancer patients, patients with benign ovarian tumors and healthy women were 1.64 ± 1.01, 1.32 ± 0.72, 0.78 ± 0.50 and 0.76 ± 0.34, respectively (Table 2). In addition, 3,144 Da m/z expressions differed significantly among the 4 groups of ovarian cancer patients, ovarian cancer relapse patients, benign ovarian tumor patients and healthy people ($P < 0.01$). However, there were no differences between benign ovarian tumor patients and healthy people ($P > 0.5$).

Differences in concentrations of CA125 detected in sera between ovarian cancer and relapsed ovarian cancer patients

The average CA125 levels in 26 ovarian cancer patients in stage III-IV and 22 relapsed ovarian cancer patients were 2,473.36 ± 2758.99 U/ml and 590.89 ± 801.57 U/ml, respectively. CA125 levels were significantly higher in ovarian cancer patients than in relapsed ovarian cancer patients. The difference in CA125 levels between ovarian cancer and relapsed ovarian cancer patients was consistent with differences in the 3,144 m/z peak.

ROC curve of 3,144 m/z in stages III/IV invasive epithelial ovarian cancers and benign ovarian tumors

ROC analysis was performed by comparing samples from 26 patients with stages III/IV invasive epithelial ovarian cancer and 35 patients with benign disorders. The ROC AUC of the 3,144 m/z peak was 0.78. The sensitivity and specificity of the 3,144 m/z peak for discriminating between malignant and benign ovarian tumors were 65.4% and 91.4%, respectively, with a cut-off value of 1.15 (Fig.2).

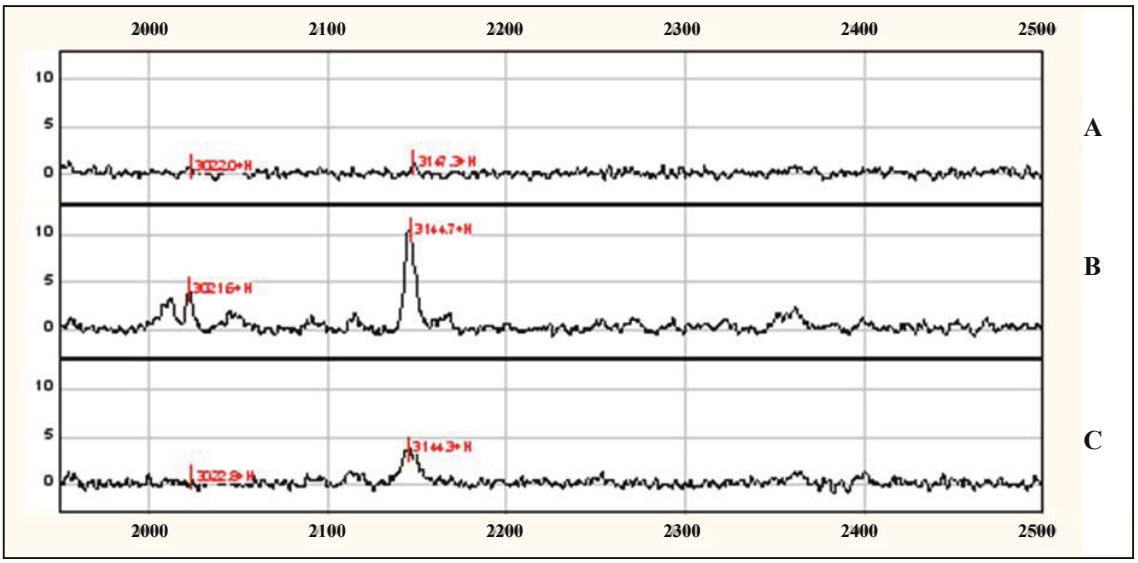

Fig.1. SELDI analysis of serum proteomic patterns in culture supernatants from HO-8910PM and HO-8910 cells. The X-axis represents the molecular mass calculation (m/z values), and the Y-axis represents relative intensity. The mass spectrographic profiles reveal relative up-regulation of m/z 3,021 and 3,144 peaks in HO-8910PM cells. A, serum free culture media; B, HO-8910PM culture supernatant; C, HO-8910 culture supernatant.

Table 2. Relative intensities of 3,144 m/z protein peaks detected in patient sera.

	Ovarian cancer			
Protein (m/z)	Stage III-IV	Relapse	Benign tumor	Healthy women
3,144	1.64 ± 1.01**	1.32 ± 0.72*	0.78 ± 0.50	0.76 ± 0.34

All values are presented as mean ± SD of relevant groups. * $P < 0.01$, ** $P < 0.001$.

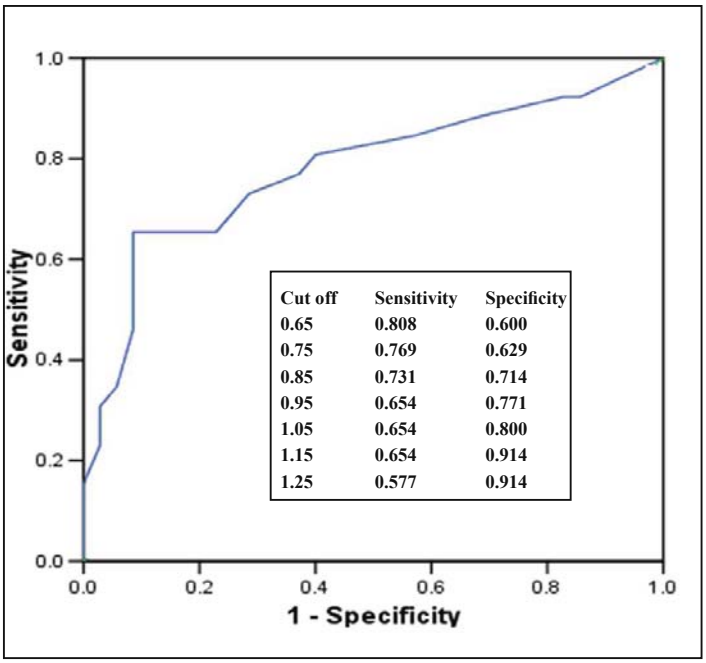

Fig.2. ROC curve of 3,144 m/z. Serum samples from 26 patients with stages III/IV invasive epithelial ovarian cancer and 35 patients with benign disorders in comparison.

Proteins identified by TagIdent (http://expasy.org/tools/tagident.html)

We performed an online TagIdent (protein database) search using the size of 3,144 m/z determined from SELDI-TOF-MS analysis. Our search criteria allowed for a 0.1% size error. We identified a CD24 fragment (Chain: 27-59, pI: 5.72, MW: 3142) as a potential candidate protein for the 3144 Da peak.

Discussion

Complex serum proteomic patterns might reflect the underlying pathologic state of organs such as the ovary. SELDI-TOF-MS, an innovative proteomic technology, has overcome many of the limitations of two-dimensional electrophoresis and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry[9]. SELDI-TOF is widely used to investigate serum proteomic patterns[10]. The use of proteomics for identifying potential diagnostic biomarkers has been explored so far in several types of cancers, including prostate[11,12], colon[13], breast[14] and ovarian cancer[4].

We discovered a distinct 3,144 m/z protein peak that was consistently present in lysis solutions and culture supernatants from HO-8910PM and HO-8910 ovarian cancer cells. It differed significantly among ovarian cancer patients, relapsed ovarian cancer patients, benign ovarian tumor patients, and healthy women. Using the online TagIdent tool, we identified a CD24 fragment (Chain: 27-59, pI: 5.72, MW: 3142) as a potential candidate protein for the 3,144 Da peak. CD24 is a small, heavily glycosylated cell surface protein, which is expressed in a large variety of solid tumors. It is thought to play an important role in tumor progression and metastasis[15-17]. CD24 expression is associated with rapid cell spreading and high degrees of cell motility and invasion[18]. In addition, CD24 is an established marker for poor prognosis in ovarian and other carcinomas[19-21].

The origin and full identity of discriminatory proteins or peptides are still under investigation. They have been detected in the low-molecular-weight serum proteome but still remain unknown at present. These proteins or peptides could be derived from the host organ or the tumor, or could constitute metabolic fragments. By comparing the differential expression of proteins in 2 human ovarian cancer cell lines with different metastatic potential, and by comparing serum proteins or peptides in ovarian cancer patients, ovarian cancer relapse patients, benign ovarian tumor patients, and healthy women, we identified several metastasis-related proteins. These proteins or peptides might be proven to be useful biomarkers for the diagnosis of ovarian cancer metastasis.

References

1 Bast RC Jr. Status of tumour markers in ovarian cancer screening. J Clin Oncol 2003; 21 (10 Suppl): 200s-205s.
2 Engel J, Eckel R, Schubert-Fritschle G, et al. Moderate progress for ovarian cancer in the last 20 years: prolongation of survival, but no improvement in the cure rate. Eur J Cancer 2002; 38: 2435-2445.
3 Ashworth A, Balkwill F, Bast RC Jr, et al. Opportunities and challenges in ovarian cancer research, a perspective from the 11th Ovarian Cancer Action/HHMT Forum, Lake Como, March 2007. Gynecol Oncol 2008; 108: 652-657.
4 Petricoin EF, Ardekani AM, Hitt BA, et al. Use of proteomic patterns in serum to identify ovarian cancer. Lancet 2002; 359: 572-577.
5 Zhang Z, Bast RC Jr, Yu YH, et al. Three biomarkers identified from serum proteomic analysis for the detection of early stage ovarian cancer. Cancer Res 2004; 64: 5882-5890.

6 Mou HZ, Xu SH, Zhang YY, et al. Establishment of a human ovarian cancer cell line (HO-8910) and its characterization. Zhonghua Fuchanke Zazhi 1994; 29: 162-164 (Chinese).
7 Xu SH, Qian LJ, Mou HZ, et al. Establishment of a highly metastatic human ovarian cancer cell line (HO-8910PM) and its characterization. J Exp Clin Cancer Res 1999; 18: 233-239.
8 Xia L, Cai WL, Zhang LJ, et al. The preliminary analysis of distinct proteins for different subtypes of lung cancer cells lines. Xiandai Yiqi 2004; 10: 13-17 (Chinese).
9 Wadsworth JT, Somers KD, Cazares LH, et al. Serum protein profiles to identify head and neck cancer. Clin Cancer Res 2004; 10: 1625-1632.
10 XU Y. Research advance of protein fingerprinting technology in laboratory diagnosis and clinical medicine. Jichu Yixue Yu Linchuang 2007; 27: 134-142 (Chinese).
11 Qu Y, Adam BL, Yasui Y, et al. Boosted decision tree analysis of surface-enhanced laser desorption/ionization mass spectral serum profiles discriminates prostate cancer from noncancer patients. Clin Chem 2002; 48: 1835-1843.
12 Petricoin EF, Ornstein DK, Paweletz CP, et al. Serum proteomic patterns for detection of prostate cancer. J Natl Cancer Inst 2002; 94: 1576-1578.
13 Lawrie LC, Curran S, McLeod HL, et al. Application of laser capture microdissection and proteomics in colon cancer. Mol Pathol 2001; 54: 253-258.
14 Li J, Zhang Z, Rosenzweig J, et al. Proteomics and bioinformatics approaches for identification of serum biomarkers to detect breast cancer. Clin Chem 2002; 48: 1296-1304.
15 Lim SC, Oh SH. The role of CD24 in various human epithelial neoplasias. Pathol Res Pract 2005;201: 479-486.
16 Sagiv E, Memeo L, Karin A, et al. CD24 is a new oncogene, early at the multistep process of colorectal cancer carcinogenesis. Gastroenterology 2006; 131: 630-639.
17 Sagiv E, Kazanov D, Arber N, et al. CD24 plays an important role in the carcinogenesis process of the pancreas. Biomed Pharmacother 2006; 60: 280-284.
18 Baumann P, Cremers N, Kroese F, et al. CD24 expression causes the acquisition of multiple cellular properties associated with tumor growth and metastasis. Cancer Res 2005; 65: 10783-10793.
19 Runz S, Keller S, Rupp C, et al. Malignant ascites-derived exosomes of ovarian carcinoma patients contain CD24 and EpCAM. Gynecol Oncol 2007; 107: 563-571.
20 Kwon GY, Ha H, Ahn G, et al. Role of CD24 protein in predicting metastatic potential of uterine cervical squamous cell carcinoma in patients treated with radiotherapy. Int J Radiat Oncol Biol Phys 2007; 69: 1150-1156.
21 Chou YY, Jeng YM, Lee TT, et al. Cytoplasmic CD24 expression is a novel prognostic factor in diffuse-type gastric adenocarcinoma. Ann Surg Oncol 2007; 14: 2748-2758.