Serum Proteomic Analysis on Invasive Cervical Cancer

Sheke Guo¹,², Yuhuan Qiao¹, Huirong Shi¹, Xianlan Zhao¹, Liuxia Li¹

1. Department of Gynaecology and Obstetrics, The First Affiliated Hospital, Zhengzhou University, Zhengzhou, Henan 450052, China
2. Department of Gynaecology and Obstetrics, Jiaozuo People’s Hospital, Jiaozuo, Henan 454100, China

Abstract: Objective. To characterize the serum proteomic pattern and its relationship with surveillance and prognosis in judgment of patients with invasive cervical cancer. Methods. A total of 166 serum samples, including group A of 49 patients with invasive cervical cancer and 71 age-matched healthy women; and group B of 49 invasive cervical cancer patients, 24 invasive cervical patients with radical hysterectomy and pelvic lymphadenectomy and 22 review patients at the 3rd month after surgery, were tested by SELDI-TOF-MS with IMAC-Cu. Group A was to build a diagnosis model and detect the significant proteins that might be potentially as biomarkers. The significant proteins from group A were compared with group B. Results. 47 proteins were detected with a significant level of P<0.01 from group A. 6 proteins with m/z value of 8929.31, 7930.52, 9127.31, 8141.01, 7963.06 and 9280.63 had high score (>95 %) in building a model of decision tree classification algorithm for invasive cervical cancer detection. The accuracy, sensitivity and specificity of m/z value of 8929.31 were 98.33%, 97.96% and 98.59% respectively. The 6 proteins, which appeared to be down-regulated in patients with invasive cervical cancer, had gradually retrieved in a level of P<0.01 after surgery, except m/z value of 9280.63, and continuously climbed in a level of P<0.01 at the time of 3 months postoperation including 9280.63. Conclusions. The six proteins as a novel group of biomarkers could potentially be used for the treatment surveillance and prognosis prediction of cervical cancer. [Life Science Journal. 2006;3(4):17-22] (ISSN: 1097-8135).

Keywords: SELDI-TOF-MS; cervical cancer; radical hysterectomy; pelvic lymphadenectomy; biomarker

Abbreviations: SELDI-TOF-MS: surface-enhanced laser desorption/ionization time-of-flight mass spectrometry

1 Introduction

Cervical cancer remains an important public health problem, ranking second only to breast cancer as the most common malignancy among women worldwide, especially in developing countries. According to Global Cancer Statistics published in 2005¹¹, the estimate annual number of new cases of cervical cancer worldwide is 492,000 and the estimated number of deaths is 274,000 in the year of 2002. However, there are only 83,000 new cases, 40,000 deaths in developed countries while 409,000 new cases and 234,000 deaths in developing countries. In general terms, it is much more common in developing countries. Routine screening has decreased the incidence of invasive cervical cancer, but invasive cervical cancer is still more common in women middle aged and older of poor socioeconomic status, who are less likely to receive regular screening and early treatment¹¹. The cause of cervical cancer is not very clear. Infection with specific subtypes of human papillomavirus has been strongly implicated in cervical carcinogenesis but HPV infection alone is insufficient for malignant transformation²³. The information regarding tumor type, grade, extent of invasion and metastasis and completeness of excision, etc were histopathologically provided. The treatment scheme and assessment of prognosis at present are based on clinical features⁴⁻¹⁰, such as clinical stage, differentiation of the cancer cells, the metastasis of pelvic lymph nodes, surgical margin involved, deep stroma invasion and parametrial extension. And the prognosis of cervical cancer patients has improved in the past decade as a result of improvements in screening programs and early detection¹¹⁻¹⁵, advanced in surgery⁴,¹⁶, radiotherapy and chemotherapy¹⁷⁻²⁰. However, not all early stage patients with cervical cancer are cured. Some cases may be recurrent or even lead to death²¹. Cervical cancer related biomarkers have not been well characterized. Protein expression in serum of patients with invasive cervical cancer should contain information about cancer development and progression. Utilization of this information for discovering biomarkers that could be used to monitor the treatment response and to predict the prognosis of cervical cancer patients could be possible if a tool can be developed to rapidly analyze and display changes in protein expression. In fact, it appears that no single
biomarker, or few specific tumor markers will be effective in improving detection, treatment, and prognosis in cervical cancer. Proteomic technologies, especially the surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) technology, are providing the tools needed to discover a group of disease-associating biomarkers\textsuperscript{22-24}. The SELDI-TOF-MS technology coupled with different protein chips facilitates protein profiling of complex biological mixtures. Therefore, the present study was undertaken to characterize the serum proteomic patterns in cervical cancer and to correlate these molecules with cervical cancer prognosis.

2 Materials and Methods

2.1 Serum samples

A total of 166 serum specimens were obtained from the department of gynecology and medical examination center, the First Affiliated Hospital, Zhengzhou University (Zhengzhou, China) from June 1, 2005 to November 31, 2005. They were divided into two groups: group A of 49 patients with invasive cervical cancer and 71 age-matched healthy women; group B of 49 invasive cervical cancer patients. 24 patients with invasive cervical cancer whose serum samples were collected on the 10th day after radical hysterectomy and pelvic lymphadenectomy and 22 review patients at the time of 3 months after operation. All consenting patients with invasive cervical cancer were histopathologically diagnosed by biopsy and reconfirmed histopathologically after operation. Serum samples of patients with invasive cervical cancer were collected from the patients without any treatment such as chemotherapy, radiotherapy, etc. All blood samples were taken after overnight fasting. The medial age of patients with invasive cervical cancer was 47 years old (25 – 75 years old) and the medial age of the control group was 45 years old (22 – 73 years old). There was no statistically significant difference in the ages between the patients and controls \(P > 0.05\). The clinical staging was according to the criteria of Federation of International Gynecologists and Obstetrician (FIGO). The clinical characteristics of 49 patients with invasive cervical cancer were listed in Table 1.

### Table 1. Patients characteristics \(n = 49\)

<table>
<thead>
<tr>
<th>FIGO stage</th>
<th>n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I b – II a</td>
<td>39 (79.59%)</td>
</tr>
<tr>
<td>&gt; II a</td>
<td>10 (20.41%)</td>
</tr>
<tr>
<td>Histopathology</td>
<td></td>
</tr>
<tr>
<td>Squamous cell</td>
<td>45 (91.84%)</td>
</tr>
<tr>
<td>Others</td>
<td>4 (8.16%)</td>
</tr>
</tbody>
</table>

2.2 Preparation of serum samples for SELDI analysis

3 ml blood sample was obtained and centrifuged with 2,000 rpm at 4 °C for 10 minutes within 30 minutes after collection. All serum samples were aliquoted into 100 µl and stored at -80 °C until use. Serum samples for SELDI-TOF analysis were prepared by vortexing 5 µl of serum with 10 µl (1:2) U9 (9 mol/L urea, 2% Chaps, 50 mmol/L Tris-HCl, pH 9.0) at 4 °C for 30 minutes, and then diluted to 1 : 12 in binding PBS buffer (pH 7.0), vortexed at 4 °C for 30 minutes. Eight-spot immobilized metal affinity capture array-Cu (IMAC-Cu) chips (Ciphergen Biosystems, Fremont, CA, USA) was put onto a bioprocessor, a device that holds chips. The spots were activated with 50 µl of 100 mmol/L CuSO\(_4\) and vortexed for 5 minutes, followed by a deionized water rinse, then 50 µl of 100 mmol/L sodium acetate buffer (pH 4.0) was added to each array and shaken for 5 minutes, followed by a deionized water rinse again. The activated array surfaces were equilibrated with 150 µl of PBS (pH 7.0), agitated for 5 minutes, twice. 50 µl of diluted sample were applied onto the array surface and shaken at 4 °C for 60 minutes. Then the chips were washed twice, with 150 µl of PBS for 5 minutes each wash cycle. The chips were removed from the bioprocessor, air-dried. Before SELDI-TOF-MS analysis, 0.5 µl of a saturated EAM solution (sinaipinic acid in 50% aqueous acetonitrile and 0.5% trifluoroacetic acid) was applied onto each spot twice and air-dried between each EAM application.

2.3 SELDI-TOF-MS analysis

The chips were placed in the PBS-II mass spectrometer reader (Ciphergen Biosystems, Fremont, CA, USA). Time-of-flight mass spectra were generated by averaging 90 laser shots at a laser intensity of 180 and a detector sensitivity of 9. The spectra were calibrated by using the All-in-1 protein molecular mass standard (Ciphergen Biosystems, Fremont, CA, USA). The reproducibility of the SELDI-TOF system was determined using two representative serum samples; one from the healthy controls and the other from the cervical invasive cancer patients, according to the manufacturer’s instructions.

2.4 Statistical analysis of SELDI-TOF-MS spectra

All spectra were compiled, and the peak intensities were normalized to the total ion current of mass to charge \((m/z)\) values from 1,500 Da to 15,000 Da using ProteinChip Software 3.2.0 (Ciphergen Biosystems, Fremont, CA, USA). The cluster data was analyzed by using Biomarker Pat-
tern Software 4.0.1 and Biomarker Wizard Software (Ciphergen Biosystems, Fremont, CA, USA). The construction of the decision tree classification algorithm with ten fold cross validation were accomplished. It creates tree-like structured decision diagrams by splitting the original dataset (parent node) into two nodes (child nodes) of highest possible purity, in which splitting decision was defined as the intensity levels of one peak. Each child node then becomes a parent node at the time of creation and can be the origin of a new split. The splitting process continued till terminal nodes. The classification of terminal nodes was determined by the group of samples (i.e. invasive cervical cancer or control) representing the majority of samples in the corresponding node. Variable importance scores reflect the contribution of each variable to classification. The variable used to split the root node was ranked as the most important. The variable received a zero score, indicating that it did not play any role in the analysis as either primary splitters or surrogates. Sensitivity was calculated as the ratio of the number of correctly classified cancer samples to the total number of cancer samples while specificity was calculated as the ratio of the number of non-cancer samples correctly classified to the total number of non-cancer samples. t test and One-Way ANOVA (SPSS Software 11.0) were used for comparison the mass peaks of group B.

3 Results

47 qualified mass peaks were identified with a significant level of \( P < 0.01 \) (Figure 1). 6 mass peaks of them with the m/z value of 8929.31, 7930.52, 9127.31, 8141.01, 7963.06 and 9280.63 were of importance as decision tree classification algorithm (classification score > 95%), which appeared to be down regulated in patients with invasive cervical cancer. The score in classification, intensity of split and the mean intensity were shown in Table 2. The decision tree model with m/z value of 8929.31 had automatically built with the least nodes and lowest ratio of mis-judged wrong classification (Figure 2). And the judgment of cancer or healthy control was made according to the rules of the model tree. This model has correct classification ratio of 98.33%, sensitivity of 97.96%, specificity of 98.59%. The mass spectrum and pseudogel view of m/z value of 8929.31 were shown in Figure 3. The intensity of this 6 mass peaks, had gradually retrieved in a level of \( P < 0.01 \) after operation, except m/z value of 9280.63 (a little lower than preoperation 0.6307 ± 0.5789/0.4339 ± 0.2940), and continuously climbed in a level of \( P < 0.01 \) at the time of 3 months postoperation including 9280.63. Comparison of intensities of this 6 mass peaks within group B was listed in Table 3. Results of \( t \) test for the 6 mass peaks of every variable in group B were shown in Tables 4 and 5.

4 Discussion

Proteins carry out most of the cellular functions. Therefore, the direct measurement of protein levels and activity within the cell is the best determinant of overall cellular function. However, as the range of protein expression and modification is dynamic, it is a clear need for high-throughput assays in proteomics. Here, the novel proteomic analytical technique referred to as SELDI technology becomes a valuable tool in determining the presence of protein within a sample. This high throughput, array-based technology can bring us closer to a better understanding of cellular functions at the protein level. It produces spectra based on the m/z of complex proteins and on their binding affinity to the chip surface[23 - 25].
Figure 3. The mass spectra and gel view of M8929.31. Y axis is the relative intensity of proteins; X, the ratio of mass to charge.

Table 2. The score and split intensity in value of classification algorithm, mean intensity and standard deviation ($\bar{x} \pm s$) of the six significant proteins in the sera of invasive cervical cancer patients and control group

<table>
<thead>
<tr>
<th>m/z</th>
<th>Score</th>
<th>Split</th>
<th>Control ($\bar{x} \pm s$)</th>
<th>Cervical cancer ($\bar{x} \pm s$)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>8929.31</td>
<td>100</td>
<td>1.864</td>
<td>19.88393 13.34943</td>
<td>0.407668 0.307375</td>
<td>0.0000</td>
</tr>
<tr>
<td>7930.527</td>
<td>98.25</td>
<td>1.427</td>
<td>4.541067 2.145074</td>
<td>0.325958 0.198719</td>
<td>0.0000</td>
</tr>
<tr>
<td>9127.317</td>
<td>98.25</td>
<td>0.746</td>
<td>4.884198 2.960881</td>
<td>0.137516 0.112587</td>
<td>0.0000</td>
</tr>
<tr>
<td>8141.019</td>
<td>98.12</td>
<td>1.506</td>
<td>5.043384 1.486190</td>
<td>0.483423 0.317135</td>
<td>0.0000</td>
</tr>
<tr>
<td>7963.060</td>
<td>97.35</td>
<td>1.406</td>
<td>3.433155 1.106128</td>
<td>0.340217 0.263501</td>
<td>0.0000</td>
</tr>
<tr>
<td>9280.430</td>
<td>97.00</td>
<td>2.650</td>
<td>7.370173 2.190772</td>
<td>0.636661 0.578872</td>
<td>0.0000</td>
</tr>
</tbody>
</table>

Table 3. Mean intensity and standard deviation ($\bar{x} \pm s$) of the six mass peaks within group B (One-Way ANOVA)

<table>
<thead>
<tr>
<th>m/z</th>
<th>Cervical cancer</th>
<th>Postoperation</th>
<th>3-month review</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>8929.31</td>
<td>0.4077 ± 0.2074</td>
<td>0.7062 ± 0.2409</td>
<td>1.6468 ± 0.6334</td>
<td>74.391</td>
<td>0.000</td>
</tr>
<tr>
<td>7930.527</td>
<td>0.3256 ± 0.1987</td>
<td>0.8375 ± 0.4328</td>
<td>2.5177 ± 1.0523</td>
<td>113.575</td>
<td>0.000</td>
</tr>
<tr>
<td>9127.32</td>
<td>0.1357 ± 0.1106</td>
<td>0.3419 ± 0.2036</td>
<td>0.5446 ± 0.2654</td>
<td>40.516</td>
<td>0.000</td>
</tr>
<tr>
<td>8141.02</td>
<td>0.4834 ± 0.3171</td>
<td>1.1481 ± 0.8371</td>
<td>2.0022 ± 0.6673</td>
<td>55.530</td>
<td>0.000</td>
</tr>
<tr>
<td>7963.06</td>
<td>0.3402 ± 0.2635</td>
<td>0.6518 ± 0.3728</td>
<td>1.6923 ± 0.4796</td>
<td>113.506</td>
<td>0.000</td>
</tr>
<tr>
<td>9280.43</td>
<td>0.6307 ± 0.5789</td>
<td>0.4339 ± 0.2940</td>
<td>1.3837 ± 0.6642</td>
<td>19.410</td>
<td>0.000</td>
</tr>
</tbody>
</table>

Table 4. Changes of the six mass peaks between invasive cervical cancer and postoperation ($t$ test)

<table>
<thead>
<tr>
<th>m/z</th>
<th>Cervical cancer</th>
<th>Postoperation</th>
<th>t</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>8929.31</td>
<td>x ± s</td>
<td>0.4077 ± 0.2074</td>
<td>0.7062 ± 0.2409</td>
<td>0.0001</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>0.0001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7930.527</td>
<td>x ± s</td>
<td>0.3256 ± 0.1987</td>
<td>0.8375 ± 0.4328</td>
<td>0.0000</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>0.0000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9127.31</td>
<td>x ± s</td>
<td>0.1357 ± 0.1106</td>
<td>0.3419 ± 0.2036</td>
<td>0.0000</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>0.0000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8141.01</td>
<td>x ± s</td>
<td>0.4834 ± 0.3171</td>
<td>1.1481 ± 0.8371</td>
<td>0.0069</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>0.0001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7963.06</td>
<td>x ± s</td>
<td>0.3402 ± 0.2635</td>
<td>0.6518 ± 0.3728</td>
<td>0.0000</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>0.0000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9280.43</td>
<td>x ± s</td>
<td>0.6307 ± 0.5789</td>
<td>0.4339 ± 0.2940</td>
<td>0.0000</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>0.0621</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 5. Comparison intensities of the six mass peaks between postoperation and review ($t$ test)

<table>
<thead>
<tr>
<th>m/z</th>
<th>Postoperation</th>
<th>Review</th>
<th>t</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>8929.31</td>
<td>x ± s</td>
<td>0.7062 ± 0.2409</td>
<td>1.6468 ± 0.633563</td>
<td>0.0000</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>0.0000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7930.527</td>
<td>x ± s</td>
<td>0.8375 ± 0.4328</td>
<td>2.5177 ± 1.0523</td>
<td>0.0000</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>0.0000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9127.31</td>
<td>x ± s</td>
<td>0.3419 ± 0.2036</td>
<td>0.5446 ± 0.2654</td>
<td>0.0069</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>0.0000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8141.01</td>
<td>x ± s</td>
<td>1.1481 ± 0.8371</td>
<td>2.0022 ± 0.6673</td>
<td>0.0000</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>0.0000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7963.06</td>
<td>x ± s</td>
<td>0.6518 ± 0.3728</td>
<td>1.6923 ± 0.4796</td>
<td>0.0000</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>0.0000</td>
<td></td>
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<td>1.3837 ± 0.6642</td>
<td>0.0000</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>0.0000</td>
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</table>
Comparisons of the protein peak patterns obtained from samples representing different status are expected to provide detailed diagnostic patterns classifying cellular or pathological status. It needs low amounts of complex biological specimens, no protein tagging and can be run automatically. Only the mass values detected both reproducibly and reliably are required to make a correct classification or diagnosis without necessary to know the identities of the masses only for the purpose of differential diagnosis. SELDI-TOF-MS technology provides a better and easier tool to identify the complex serum protein profiling. This technology has been successfully applied for analyzing protein expression in several kinds of cancer, such as breast cancer[26], prostate cancer[27], cancer of digestive system[28-30], gynecologic cancer[31-33], etc. Most of those studies demonstrated the diagnostic ability of SELDI for protein profiles and its potential utility for cancer detection and diagnosis.

To our best knowledge, cervical cancer has not been found potential biomarkers, which can be used for detection, diagnosis, treatment surveillance and prognosis prediction. Treatment surveillance and prognosis prediction has played a pivotal role in a complete treatment scheme of patients with cervical cancer. It could not only rely on the experiences of gynecologists and oncologists but also on some objective signs such as biomarkers. In this study, 6 peaks were identified as the potential biomarkers with a significant level of P < 0.01 and a significant score in a decision tree classification algorithm with high sensitivity and specificity. The 6 mass peaks, down regulated in patients with invasive cervical cancer, were slowly retrieved after operation except m/z value of 9280.43, a little lower than preoperation (P > 0.05). Afterwards they were continuously climbed in a level of P < 0.01 including m/z of 9280.43 until 3-month review after operation. However, they were still more less than in healthy women even at the time of 3-month review. So they would be thought as a group of protective factors or tumor-suppressors, proteins or peptides. They are of importance in the initiation, progression of cervical cancer. Therefore it would be possible that the 6 mass peaks could be used as novel potential biomarkers for monitoring and assessing the treatment effect, predicting the prognosis of invasive cervical cancer. If one or more of them is declined or keeps same level after treatment, the treatment scheme for patients with cervical cancer would not be ideal or the remaining cancer cells would be proliferating. Based on this, we may proceed with further studies using this SELDI-TOF-MS technology in a large population, particularly in review patients with formal treatment for invasive cervical cancer, at least up to the 5-year survival year. And the further efforts would be invested in purifying, identifying and characterizing these proteins or peptides for better understanding what biological role these proteins or peptides may play in the carcinogenesis of cervical cancer. So their exact identities will be possible to find a more simple way to test them for clinical uses.

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Correspondence to:
Yuhuan Qiao, M.D.
Department of Gynaecology
The First Affiliated Hospital
Zhengzhou University
Zhengzhou, Henan 450052, China
Telephone and Fax: 86-371-6699-9848

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