Basic Research Paper

Screening of differentially expressed proteins associated with directional highly lymphatic metastasis in ovarian carcinoma cell lines using SELDI-TOF-MS technology

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Key words: directional lymphatic metastasis, protein chip, differentially expressed protein, surface-enhanced laser desorption and ionization, time of flight-mass spectrometry

Background and Objective: The ovarian serous papillary adenocarcinoma cell line SKOV3 and its subclones SKOV3-pm2 and SKOV3-pm3 are cell models to investigate the molecular mechanism of invasion and metastasis of ovarian cancers. This study screened differentially expressed proteins between ovarian carcinoma cell lines with directional (SKOV3-pm2 and SKOV3-pm3) and non-directional (SKOV3) highly lymphatic metastasis potentials using time-of-flight mass spectrometry technology and protein chips. Methods: The lymphatic metastasis rates of the three cell lines were detected in animal models. Proteins in endochylema and supernatants of the three cell lines were screened using surface-enhanced laser desorption and ionization-time of flight-mass spectrometry (SELDI-TOF-MS). Each sample was examined using weak cation exchange (CM-10) protein chip assay and immobilized metal affinity capture (IMAC-3) SELDI ProteinChip array. Detected protein peaks were filtrated and analyzed using Ciphergen proteinchip software 3.2.0 and Biomarker Wizard software. Differentially expressed proteins were defined as those whose absolute ratio values were greater than 0.5. Results: Lymphatic metastasis rates in SKOV3, SKOV3-pm2 and SKOV3-pm3 cell xenografts in nude mice were 20%, 90% and 100%, respectively (p < 0.05). Proteins in endochylema with a mass-to-charge ratio (m/z) were 6,971, 7,475, 9,089, 9,453, 10,103, 11,655, and the protein in supernatants whose m/z was 4,746 were differentially expressed in SKOV3, SKOV3-pm2 and SKOV3-pm3 cells. Conclusion: Combined with weak cation exchange protein chip assay and immobilized metal affinity capture SELDI ProteinChip array, SELDI-TOF-MS technology can be used to screen and identify differentially expressed proteins associated with directional highly lymphatic metastasis in ovarian carcinoma cell lines.

Recently, the incidence of ovarian cancers among female reproductive system diseases tends to increase year after year and its mortality ranks the top among all gynecologic tumors. This may mainly be due to the occurrence of extensive intraperitoneal metastases that make cancer eradication difficult; in particular, retroperitoneal lymph node metastasis is a major factor causing post-operational recurrence of ovarian cancer and influencing prognosis. The majority of ovarian tumors originate in the epithelium, with the lymphatic ducts as their major path for invasion and metastasis. The total rate of positive lymph node metastasis in ovarian cancer reaches 50% and homes of the first gynecological tumors. SKOV3 is a human ovarian serous papillary adenocarcinoma cell line. In preliminary studies, we had generated subclones of this cell line from the lymphatic metastases of nude mice bearing human SKOV3 cells, specifically we screened and established two directional highly lymphatic metastasis subclones: SKOV3-pm2 as the second generation and SKOV3-pm3 cells as the third generation. Animal xenograft experiments showed that compared to primary SKOV3 cells, SKOV3-pm2 and SKOV3-pm3 formed tumors earlier, had shorter tumor doubling time, faster metastasis and a higher rate of lymph node metastasis to a broader range of locations.1

Previous research has investigated the mechanisms of lymphatic invasion by comparing the differentially expressed genes between directional (SKOV3-pm2, SKOV3-pm3) and non-lymphatic directional metastasis ovarian cancer cell lines (SKOV3) using gene chip technology,2 but gene function still needs to be tested and confirmed at the protein level. The emergence of differential proteomics provides a reliable technological platform for the...
screening of cancer metastasis-associated protein markers through identification, quantification and characterization of differentially expressed proteins. Using a novel differential proteomics technology, the surface-enhanced laser desorption and ionization-time of flight-mass spectrometry (SELDI-TOF-MS), our study detected the protein fingerprint patterns of the above three cell lines with different lymphatic directional metastasis abilities. We applied a simple, efficient and sensitive way to screen for proteins associated with invasive potentials in order to provide a research base for further investigation of the mechanisms for specific lymph node metastasis in ovarian cancer.

Materials and Methods

Materials. Major reagents and instruments. Acetonitrile (CAN), trifluoroacetic acid (TFA), urea, energy absorb molecule-sinapinic acid (SPA), N-2-hydroxyethylpiper-azine-N-2-ethanesulfonic acid (HEPES), 3-(3-Cholamidopropyl) dimethylammonio-1-propanesulfonic acid (CHAPS), Tris-(hydroxymethyl) aminomethane-hydrochloric acid buffer (Tris-HCl), dithiothreitol (DTT), protease inhibitors, CuSO₄₃, sodium acetate, HCl and HPLC water were chromatography grade products from Sigma-Aldrich, USA. ProteinChip Biology System II C (PBS II C) protein chip reader and accessory CM-10 weak cation exchange chips and IMAC3 immobilized metal affinity capture chips were purchased from Ciphergen, USA.

Cell lines and experimental animals. human ovarian serous papillary adenocarcinoma cell line SKOV3 was purchased from the Cancer Institute of Chinese Academy of Medical Sciences and passed by Guangxi Cancer Institute; directional lymphatic metastasis second generation subclone SKOV3-pm2 and third generation subclone SKOV3-pm3 were established and preserved by the Clinic Experimental Center of Guangxi Cancer Institute.1 Experimental animals were all NIH lineage of nude mice (NU/NU), purchased from Guangxi Medical University Experimental Animal Center, certification number of SCXX-Gui-No.2003-003, aged 4–6 weeks, weight 20–25 g, female, kept on laminar airflow, aged 4–6 weeks, weight 20~25 g, female, kept on laminar airflow, liquid was discarded and the same procedure was repeated once. 100 μL protein samples were diluted with conjugate buffer, added into each well (final loading concentration of 1.0 μg/μL for cytosolic proteins and 0.8 μg/μL for secretory proteins) and incubated at room temperature for 60 min with oscillation. The solution was then discarded, rinsed twice by conjugate buffer, washed by PBS three times, replaced medium and continued to culture for another 24 h. Cell culture medium of the same type of cells was collected in centrifuge tubes and centrifuged at 10,000 xg low-temperature for 30 min. Supernatant was then placed into ultrafilter centrifuge tubes (cut-off molecular weight of 3 ku), centrifuged at 5,000 xg low temperature until concentrated to about 20 times, aliquoted and preserved at -80°C. Bradford assay was applied to determine protein concentration.

Extraction of secretory proteins in cultured cells. SKOV3, SKOV3-pm2 and SKOV3-pm3 cells were cultured till about 90% confluence, washed by PBS three times, replaced medium by serum-free RPMI-1640 and continued to culture for another 24 h. Cell culture medium of the same type of cells was collected in centrifuge tubes and centrifuged at 10,000 xg low-temperature for 30 min. Supernatant was then placed into ultrafilter centrifuge tubes (cut-off molecular weight of 3 ku), centrifuged at 5,000 xg low temperature until concentrated to about 20 times, aliquoted and preserved at -80°C. Bradford assay was applied to determine protein concentration.

Extraction of cytosolic protein. Cell lysis buffer was thawed on ice, added into culture flasks at 10–14 μL/cm², incubated on ice for 30 min, collected cell lysate of the same type of cells, centrifuged at 10,000 xg, low-temperature for 30 min, aliquoted supernatant and saved at -80°C. Bradford assay was applied to determine protein concentration.

Procedure of IMAC-3 protein chip experiment. IMAC3 chip was carefully taken out, marked on the back by time and chip type, 50 μL CuSO₄₃ (100 mmol/L) was added into each well, placed into air bath shaker, incubated at room temperature for ten min (200 r/min), CuSO₄₃ was poured out added rinsed by HPLC water and dried; 50 μL sodium acetate was added (100 mmol/L pH 4.0) into each well, incubated at room temperature for five min with oscillation (200 r/min), unbound Cu ions were removed from chips, rinsed by HPLC water and dried. IMAC3 chips were placed onto the bio-chip processor (Bioprocessor), 200 μL IMAC-3 chip conjugate buffer (100 mmol/L NaCl, pH 7.0) was added into each well, incubated at room temperature for five min with oscillation, liquid was discarded and the same procedure was repeated once. 100 μL protein samples were diluted with conjugate buffer, added into each well (final loading concentration of 1.0 μg/μL for cytosolic proteins and 0.8 μg/μL for secretory proteins) and incubated at room temperature for 60 min with oscillation. The solution was then discarded, rinsed twice by conjugate buffer, was shaken at room temperature for five min and then shower-rinsed chips by HEPES (1 mmol/L, pH 7.0) for 30 s. The Bioprocessor was unloaded, 200 μL HPLC water was added into each well and quickly dried. After chips surface air dried, 0.5 μL saturated SPA was added into every spot, slightly dried, repeated once, and finally protein chip reader (PBS II C) was used to detect and analyze protein spectrum.

Procedure of CM-10 protein chip experiment. The CM-10 chip was carefully taken out, marked with time and chip type on the back, placed onto the bio-chip processor (Bioprocessor), 200 μL CM-10 chip conjugate buffer (100 mmol/L sodium acetate, pH 4.0) was added onto each spot to activate, incubated at room temperature for five min with oscillation, the solution was discarded and repeated once. Protein samples (100 μL) diluted...
with conjugate buffer were added into each well (final loading concentration of 1.0 μg/μL for cytosolic proteins and 0.8 μg/μL for secretory proteins) and incubated at room temperature for 60 min with oscillation. The solution was then discarded, rinsed twice by conjugate buffer and shaken at room temperature for five min. The Bioprocessor was unloaded, 200 μL HPLC water was added into each well and quickly dried. After chips surface air dried, 0.5 μL saturated SPA was added into each spot, slightly dried, repeated once, and finally protein chip reader (PBS II C) was used to detect and analyze protein spectrum.

Data collection and results analysis. The cytosolic and secretory proteins were extracted from SKOV3, SKOV3-pm2 and SKOV3-pm3 cell lines and IMAC-3 and CM-10 protein chip were used to perform screening tests. PBS II C protein chip reader was applied to read the chip information and initial laser intensity for detection was 185, with detector sensitivity of 8. We used Ciphergen Protein Software 3.2.0 for automatic data collection, rapidly and accurately drew protein mass spectrum, in which protein peak intensity was set as ordinate and protein mass to charge ratio (m/z) was set as abscissa. Protein peaks with signal/noise ratio (s/n) >5 and m/z between 2,000~50,000 were collected. Differentially expressed proteins were defined by difference in protein peak intensity for more than 0.5-fold. Since this result might be affected by metal ions and the matrix, we excluded any protein peaks with an m/z value less than 2,000. Detected valid protein peaks were analyzed using Biomarker Wizard software from Ciphergen, USA, which can quickly calculate the p value between experimental group and control group for proteins with the same m/z value determined by Ciphergen Protein Software 3.2.0, and the molecular weight and relative intensity of the differentially expressed proteins were exported.

Statistical methods. The number of lymph node metastasis occurring in nude mice after subcutaneous xenograft of SKOV3, SKOV3-pm2 and SKOV3-pm3 cells was recorded. Results were presented as incident rate, which consisted of quantitative data from multiple groups. SPSS13.0 software was used for statistical analysis, and comparison between the rate of each group was done by χ² test. p < 0.05 was set as the criteria for statistical significance.

Results

Lymph nodes metastasis rate. The lymph nodes metastasis rate of non-directional metastasis cell line SKOV3 at peripheral tumor cells grafting site, ipsilateral superficial inguinal lymph nodes and abdominal aortic lymph nodes were respectively 20% (2/10), 90% (9/10) and 100% (10/10), which were all statistically different (p < 0.05) from that of the directional highly metastasis cell line SKOV3-pm2 and SKOV3-pm3. This result was in compliance with the requirement for individual cell model in this study (Table 1).

Differentially expressed cytosolic proteins between directional lymphatic metastasis (SKOV3-pm2, SKOV3-pm3) and non-directional metastasis ovarian cancer (SKOV3) cell lines. Ciphergen ProteinChip 3.2.0 software used IMAC-3 protein chip to detect cytosolic proteins extracted from directed lymphatic metastasis (SKOV3-pm2, SKOV3-pm3) and non-directional metastasis ovarian cancer (SKOV3) cell lines and Biomarker Wizard Software to analyze and display protein peaks. Results showed that four differentially expressed protein peaks existed in the protein spectra of those two groups. Their m/z values were 6,971, 7,475, 9,089 and 9,453 respectively. Compared to SKOV3 cells, those proteins in SKOV3-pm2 and SKOV3-pm3 cell lines were either upregulated or downregulated at certain degree (p < 0.05) (Fig. 1); at contrast, cytosolic proteins in these three cell lines detected by CM-10 protein chip, after being analyzed by Biomarker Wizard software, showed only two differentially expressed proteins in the spectrum. Their m/z values were 10,103 and 11,655 respectively (p < 0.05). Furthermore, the trend of protein upregulation followed the progression of cell invasive ability in the above three cell lines (Fig. 2).

Differentially expressed secretory proteins between directional lymphatic metastasis (SKOV3-pm2, SKOV3-pm3) and non-directional metastasis ovarian cancer (SKOV3) cell lines. Ciphergen ProteinChip 3.2.0 software used IMAC-3 protein chip to detect secretory proteins extracted from directed lymphatic metastasis (SKOV3-pm2, SKOV3-pm3) and non-directional metastasis ovarian cancer (SKOV3) cell lines and Biomarker Wizard Software to analyze and display protein peaks. Results identified one differentially expressed protein peak in the protein spectra of those two groups, with m/z value of 4,746 (p < 0.05) (Fig. 3); consistently, secretory proteins in these three cell lines detected by CM-10 protein chip, after being analyzed by Biomarker Wizard software, identified the same differentially expressed protein in the spectrum with m/z value of 4,746 (p < 0.05). Compared to SKOV3 cells, this protein in SKOV3-pm2 and SKOV3-pm3 cell lines was downregulated (Fig. 4).

Data analysis result of detected differentially expressed proteins. Through detecting the cytosolic and secretory protein spectra in directional lymphatic metastasis (SKOV3-pm2, SKOV3-pm3) and non-directional metastasis ovarian cancer (SKOV3) cell lines by CM-10 and ICAM-3 protein chips, a total of seven differen-

### Table 1  Lymphatic metastasis in each generation of SKOV3 cell transplanted nude mice

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Nude mice (n)</th>
<th>Nude mice with lymphatic metastasis (n)</th>
<th>Lymphoid node</th>
<th>Metastatic site</th>
<th>Metastatic rate (%)</th>
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<tbody>
<tr>
<td>SKOV3</td>
<td>10</td>
<td>2</td>
<td>Popliteal fossa, fold inguen</td>
<td></td>
<td>20</td>
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<tr>
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<td>9</td>
<td>Popliteal fossa, fold inguen, lateroabdominal aorta</td>
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<td>90</td>
</tr>
<tr>
<td>SKOV3-pm3</td>
<td>10</td>
<td>10</td>
<td>Popliteal fossa, fold inguen, lateroabdominal aorta</td>
<td></td>
<td>100</td>
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</table>
Screening of differentially expressed proteins associated with directional highly lymphatic metastasis in ovarian carcinoma cell lines using SELDI-TOF-MS technology

As a novel investigation method of differential proteomics, SELDI-TOF-MS protein chip technology can selectively capture proteins in the sample specific to different surface modification features of the protein chip (such as hydrophobicity and hydrophilicity, cation and anion etc.). It is simple, rapid, sensitive and high throughput, as well as only requiring small raw amounts of samples. This study used SELDI-TOF-MS technology to detect protein spectra of directed lymphatic metastasis (SKOV3-pm2, SKOV3-pm3) and non-directional metastasis ovarian cancer (SKOV3) cell lines. Based on the different chemistry and charge of the sample protein, we applied CM-10 weak cation exchange chip and IMAC-3 immobilized metal affinity chip to jointly screen for differentially expressed proteins. By comparing the protein spectra we identified eight protein peaks with significant changes in three ovarian cancer cell lines. CM-10 weak cation exchange chip has weak anion carboxyl group conjugated to the surface so it can interact with the positively charged groups on the surface of the sample to be analyzed (for example, arginine, histidine and lysine) to capture their positively charged proteins.

Discussion

The origin and development, as well as the invasion and metastasis of malignant tumors, is a complicated sequential process involving multiple factors and multiple steps. It involves the mutual modulation and antagonism among a variety of signal pathways and is accompanied by the expression alteration of many genes and their protein products. Proteomics analysis technology is of high sensitivity and specificity, and therefore helps to catch the subtle molecular changes in the process of tumor evolution, which makes it an important clue for the diagnosis and monitoring of tumor progression. In particular, the emergence of differential proteomics analyzes and compares the change and difference in overall intracellular proteins under different conditions, and furthermore identifies, quantifies and characterizes the differentially expressed proteins, which is very useful in screening tumor-associated specific proteins, new drug targeting proteins or specific markers for the diagnosis, treatment and prognosis of cancer.

As a novel investigation method of differential proteomics, SELDI-TOF-MS protein chip technology can selectively capture proteins in the sample specific to different surface modification features of the protein chip (such as hydrophobicity and hydrophilicity, cation and anion etc.). It is simple, rapid, sensitive and high throughput, as well as only requiring small raw amounts of samples. This study used SELDI-TOF-MS technology to detect protein spectra of directed lymphatic metastasis (SKOV3-pm2, SKOV3-pm3) and non-directional metastasis ovarian cancer (SKOV3) cell lines. Based on the different chemistry and charge of the sample protein, we applied CM-10 weak cation exchange chip and IMAC-3 immobilized metal affinity chip to jointly screen for differentially expressed proteins. By comparing the protein spectra we identified eight protein peaks with significant changes in three ovarian cancer cell lines. CM-10 weak cation exchange chip has weak anion carboxyl group conjugated to the surface so it can interact with the positively charged groups on the surface of the sample to be analyzed (for example, arginine, histidine and lysine) to capture their positively charged proteins.

Figure 1. Representative images of SELDI-TOF-MS and simulated electrophoresis of differentially expressed proteins in endochylema detected by protein chip IMAC3.

Figure 2. Representative images of SELDI-TOF-MS and simulated electrophoresis of differentially expressed protein in endochylema detected by protein chip CM-10.
Screening of differentially expressed proteins associated with directional highly lymphatic metastasis in ovarian carcinoma cell lines using SELDI-TOF-MS technology

Furthermore, functional protein regulating the ovarian cancer metastasis may become the drug targeting protein to treat ovarian cancer. However, the differentially expressed proteins identified in this study still need to be verified using corresponding serum or tissue samples.

Proteomics technology is still in the process of development and improvement and SELDI-TOF-MS technology also needs to be further strengthened in many technological aspects including repeatability, stability and data comparability among different laboratories. Among the three differentially expressed protein peaks detected by CM-10 chip, one was downregulated and two were upregulated in SKOV3-pm2/SKOV3-pm3 cells. IMAC-3 immobilized metal affinity chip has NAT on its surface as the active site, which can chelate divalent metal cations (such as Cu²⁺, Ni²⁺ and Ca²⁺, etc.), so that histidine, cysteine and phosphorylated amino acids in sample protein can bind onto chip surface through interacting with chelated metal ions. Among the five differentially expressed proteins captured by IMAC-3 chip, two were downregulated and three were upregulated in SKOV3-pm2/SKOV3-pm3 cells.

The above results showed that, compared to non-lymphatic directional ovarian cancer cell line SKOV3, the directional metastasis cell lines (SKOV3-pm2 and SKOV3-pm3) had some protein expression upregulation or downregulation. Although all three cell lines are ovarian cancer, the difference in biological behavior—particularly invasion and metastasis characteristics—determined the great difference in protein expression among those three cell lines. So the altered proteins can be used as markers for clinical diagnosis of ovarian cancer and to predict prognosis.

Furthermore, functional protein regulating the ovarian cancer metastasis may become the drug targeting protein to treat ovarian cancer. However, the differentially expressed proteins identified in this study still need to be verified using corresponding serum or tissue samples.

Proteomics technology is still in the process of development and improvement and SELDI-TOF-MS technology also needs to be further strengthened in many technological aspects including repeatability, stability and data comparability among different laboratories. In addition, this study captured targeting proteins based on the reactivity of different chips, which may cause the loss of protein with incompatible reactivity and therefore can not specifically bind onto the protein chip. Meanwhile, each m/z value may correspond to a number of peptides with similar molecular weight, and protein samples separated and screened by protein chips can not be recovered for further verification, so the structure and function of the differentially expressed protein, or whether it is a known protein, can not be confirmed. Therefore, chromatography purification of the target protein still needs to be performed and traditional protein identification

Figure 3. Representative images of SELDI-TOF-MS and simulated electrophoresis of differentially expressed proteins in supernatants detected by protein chip CM-10.

Figure 4. Representative images of SELDI-TOF-MS and simulated electrophoresis of differentially expressed proteins in supernatants detected by protein chip ICAM-3.
methods need to be applied to analyze the peptide fragments of purified protein and obtain its peptide fingerprint spectrum to at last compare and search for the matched protein in the protein database.\(^8\) In addition, this study was done using in vitro cell models, so in order to verify the specificity and sensitivity of the selected differentially expressed proteins, further tests need to be performed using corresponding serum or tissue samples. In future studies we will continue to purify the selected marker proteins and investigate their functions in order to provide new thoughts and methods for the clinical molecular diagnosis of ovarian cancer and its extensive intraperitoneal lymphatic metastasis.

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**References**


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**Table 2**

<table>
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<tr>
<th>m/z</th>
<th>Protein type</th>
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<th>Average intensity Directional</th>
<th>(P)</th>
<th>Regulation of the protein</th>
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