Evaluation of the effectiveness of a chemoprevention model of pancreatic adenocarcinoma using protein chip technology

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Abstract. A serum-specific protein ‘fingerprint’ model was established which is capable of evaluating the effect of chemotherapy (gemcitabine) of pancreatic adenocarcinoma. We used SELDI-TOF-MS coupled with CM10 chips and bioinformatics tools to analyze a total of 45 mouse serum samples from three groups: the healthy control group, the pancreatic cancer model group (orthotopic transplantation model of human pancreatic adenocarcinoma) and the gemcitabine-treated group to establish diagnostic models. As a result, the test set yielded a specificity of 95.0% and a sensitivity of 95.0% for pattern 1, which distinguished pancreatic adenocarcinoma from healthy individuals and a specificity of 95.0% and a sensitivity of 75.0% for pattern 3, which distinguished healthy controls, PC model group and gemcitabine-treated group, as evaluated by leave-one-out cross-validation. We concluded from this study that the SELDI-TOF-MS technique combined with bioinformatics approaches can facilitate evaluating the effect of chemotherapy (gemcitabine) for pancreatic adenocarcinoma and could be used as a potential prognostic monitoring method.

Introduction

Pancreatic cancer (PC) is among the most deadly cancers due to its aggressiveness and early metastasis. In USA, it is the fourth leading cause of cancer deaths with a five-year survival rate of 4-5% (1). Surgical resection is the only opportunity for cure and chemotherapy offers possibilities to restrict the generalization and suppress the local recurrence of the primary tumor (2). However, there are still no generally accepted methods with adequate sensitivity and specificity to evaluate the effectiveness of chemotherapy or to predict the prognosis after chemotherapy. CT is the most commonly used method in pancreatic cancer diagnosis and recurrence monitoring. But the accuracy of the diagnosis is always hampered by the desmoplastic stroma reaction (3) and difficult to distinguish malignant masses from local inflammation and fibrosis.

Many trials have analyzed the tumor monitoring effect of CA19-9 in patients with PC receiving chemotherapy and tried to make a correlation between chemotherapy response and the expression level of CA19-9 (4,5). Although a declined level of CA19-9 could be observed in 80% of PC patients who achieved a radiological complete response (CR) or partial response (PR) to gemcitabine, the decline could also be detected in 66% of patients with disease stabilization (DS) and 42% of patients with progressive disease (PD) (6), and 19% of CR patients remained normal level of CA19-9 (7,8). Thus, the predictive value of CA19-9 level in chemotherapeutic effects was low (57.6% of sensitivity and 59.0% of specificity), and there is still lack of an accurate marker by which to assess the chemotherapeutic effects of PC or to evaluate the prognosis of PC patients.

Advances in the proteomics study have introduced novel techniques for the screening of new biomarkers (9,10). ProteinChip™ technology (Ciphergen Biosystems, Fremont, CA, USA) coupled with surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) has offered a sensitive, high throughput and rapid way for screening protein expression within complex biological specimens (11,12). Previous studies have suggested specific protein ‘fingerprint’ models were more sensitive and specific than CA19-9 and are preferable in the diagnosis of PC (13-15), but the model for predicting the chemotherapeutic effect of PC is still lacking.

Using SELDI-TOF-MS with CM10 ProteinChip (weak cation exchanger) arrays to analyze serum samples of mice xenograft PC chemoprevention models (gemcitabine treated); we have identified new potential biomarkers and established protein ‘fingerprint’ models for effective chemoprevention, which could distinguish PC models from gemcitabine-effective PC models with relatively high accuracy and offers a new way to evaluate the outcomes of the chemoprevention of PC.

Materials and methods

Cell lines and animals. PANC-1 cells (American Type Culture Collection, Manassas, VA) were propagated in Dulbecco's...
For the PC model and gemcitabine-treated groups, 1 mm³ was around 1 cm³, tumor mass was taken out and minced into respective mouse. Three weeks later, when the size of tumor (9x10⁶/ml) was subcutaneously injected into the left flank of cancer.

**Hypodermic inoculation:** 0.2 ml of cell suspension Orthotopic transplantation model of human pancreatic which was controlled by qualified staff in the Zhejiang animals were maintained on daily 12-h light/12-h dark cycle, pieces of approximately 1 mm³ for use in transplantation. Animals were housed in a sterile environment, cages and water were autoclaved, bedding and food was γ-ray-sterilized. All animals were maintained on daily 12-h light/12-h dark cycle, which was controlled by qualified staff in the Zhejiang University Laboratory Animal Center.

**Orthotopic transplantation model of human pancreatic cancer.** Hypodermic inoculation: 0.2 ml of cell suspension (9x10⁶/ml) was subcutaneously injected into the left flank of respective mouse. Three weeks later, when the size of tumor was around 1 cm³, tumor mass was taken out and minced into pieces of approximately 1 mm³ for use in transplantation. Nude mice were anesthetized with pentobarbital sodium solution (Sinopharm Chemical Reagent, Beijing) via intraperitoneal injection (45 mg/kg). A left lateral laparotomy was performed, spleen and distal pancreas were mobilized. For the PC model and gemcitabine-treated groups, 1 mm³ tumor piece was then anchored to the posterior surface of each pancreas, with 6-0 absorbable transmural suture. For the healthy control group, 10 μl sodium chloride solution was injected under the pancreatic capsule as a substitute. The pancreas was then returned to the peritoneal cavity and the abdominal wall and the skin was closed with 6-0 absorbable suture.

**Experimental chemotherapy.** Gemcitabine (Lilly, France A283463) was freshly prepared in sterile water. A week after transplantation, mice in gemcitabine-treated group were intraperitoneally-injected with gemcitabine (240 mg/kg) weekly for a total of 4 times. The mice in PC model and healthy control groups were injected accordingly with isotonic sodium chloride solution.

**Specimen sampling.** Mice were weighed every week and tumor formation was checked by palpation. On the 35th day after surgery, mice from each group were sacrificed. Serum samples were collected from postorbital sinus venous, centrifuged at 3,000 rpm for 10 min and then stored at -80°C. Tumor masses from each group were carefully weighted and measured. Pathological diagnoses of all the masses were confirmed independently by two pathologists.

**Reagents and instruments.** Ciphergen SELDI-TOF-MS (PBS-II plus) and CM10 ProteinChip (weak cation exchanger) were purchased from Ciphergen Biosystems (USA). Sinapinic acid (SPA) was purchased from Fluka (USA). All other reagents were purchased from Sigma (USA).

**ProteinChip array analysis.** All serum specimens were thawed in wet ice and centrifuged at 10,000 rpm for 4 min at 4°C. The supernatants were retained. U9 buffer (10 μl) (9 M urea, 2% CHAPS, 1% DTT) was added to 5 μl of each serum sample in a 96-well cell culture plate, which was then agitation on a platform shaker at 4°C for 30 min. Next, 185 μl of sodium acetate (100 mM, pH 4.0) was added to the U9/serum mixture and was further agitation on a platform shaker at 4°C for 2 min. CM10 chips were activated by adding 200 μl of sodium acetate and agitation for 5 min twice. Diluted samples (100 μl) were applied to each spot of the bioprocessor (Ciphergen Biosystems) that contains the ProteinChip arrays. The bioprocessor was then sealed and agitation on a platform shaker for 60 min at 4°C. The excess of serum mixtures was discarded. The chips were then washed three times with 200 μl of sodium acetate and another two times with deionized water. Finally, the chips were removed from the bioprocessor and air-dried. Prior to the SELDI-TOF-MS analysis, 1 μl of a saturated solution of SPA in 0.5 l/l CAN and 5 ml/l trifluoroacetic acid was applied onto each chip twice and the chips were again air-dried.

Chips were analyzed by the PBS-II plus mass spectrometer reader (Ciphergen Biosystems). Data were obtained by averaging of 140 laser shots with an intensity of 170, a detector sensitivity of 5, a high mass of 100,000 Da and an optimized range of 1,500-20,000 Da. Mass accuracy was calibrated by the all-in-one peptidomolecular mass standard (Ciphergen Biosystems).

**Bioinformatics and biostatistics.** To establish a new diagnostic model which is capable of evaluating the effect of chemotherapy (gemcitabine) for PC, we attempted to identify three differential patterns of PC biomarkers as follows: pattern 1, healthy controls versus PC model group; pattern 2, PC model group versus gemcitabine-treated group; and pattern 3, which distinguish healthy controls, PC model group and gemcitabine-treated group at one time.

The data analysis was implemented by the Zhejiang University Cancer Institute ProteinChip Data Analysis System (ZUCI-PDAS, www.zlzx.net), which was designed by Jiekai Yu on the MATLAB Web Server 1.2.4 (The MathWorks Inc., Natick, MA, USA). The first step of our data analysis was to use the undecimated discrete wavelet transform (UDWT) method to denoise the signals. The UDWT method was based on Version 2.4 of the Rice Wavelet Toolbox (RWT). Second, the spectra were subjected to baseline correction by aligning with a monotonic local minimum curve and mass calibration (adjusting the intensity scale according to three labeled peaks that appears in all the selected spectra). Finally, to match peaks across spectra, we pooled the detected peaks if the relative difference in their mass sizes was not >0.3%. The minimal percentage of each peak, appearing in all the spectra, was specified to 10. The matched peak across spectra was defined as a peak cluster. If a spectrum did not have a peak within a given cluster, the maximal height within the cluster would be assigned to its peak value. The normalization was performed only with the identified peak clusters. To distinguish between the different
groups of data, we used a non-linear support vector machine (SVM) classifier, originally developed by Vladimir Vapnik (16), with a radial based function kernel, a parameter $\gamma$ of 0.6, and a cost of the constrain violation of 19. The leave-one-out cross-validation approach was applied to estimate the accuracy of this classifier. This approach took out one sample each time as the test set and keeps the remaining samples as the training set. This process was repeated until each sample had been taken once as a test sample. SVM classifier was based on the shareware program OSU_SVM v.3.00 Toolbox of Junshui Ma and Yi Zhao.

The capability of each peak in distinguishing different groups of data was estimated by the $p$-value of Wilcoxon t-test. The top ten peaks with the smallest $p$-value were selected for further analysis. Combinations with the highest accuracy in distinguishing different groups of data were selected as potential biomarkers. The SVM model with the highest Youden index was selected as the model. All these bioinformatics studies were integrated in the ZUCIPDAS available at www.zlzx.net.

Results

Reproducibility of the experiment. To examine the variation in data collection between the protein chips used in these experiments, quality control samples (7 serum samples from one single healthy mouse) were applied to each chip in a random fashion. The coefficient of variance (CV) for peak intensity was calculated using 10 randomly chosen peaks with a signal/noise ratio >5 and m/z <20 kD. The CV of the selected peaks, after being normalized by the intensity, was 15.4% and the CV of the selected peak masses was 0.04%.

Local growth of pancreatic xenografts. The yield of the orthotopic transplantation of human pancreatic cancer in nude mice was 100%. In accordance with previous reports (12), the tumor masses in gemcitabine-treated group were significantly smaller than the PC model group (Fig. 1). Big (1-1.5 cm in diameter) solid mass extensively replaced the pancreas was found in most cases of the PC model group, and invaded in some cases also neighboring organs; while a dramatic mass shrinkage (0.5-0.8 cm) was observed in gemcitabine-treated group. There was no change or only small cysts located in pancreas could be found in healthy control group (Fig. 2). The tumor inhibition ratio [(average volume of PC model group - average volume of gemcitabine-treated group/average volume of PC model group) x 100%] was 48.11%.

Healthy controls versus PC model group (pattern 1). A total of 186 qualified peaks were selected after noise filtering and peak cluster identification. These peaks were ranked according to the $p$-value of Wilcoxon rank sum test. The top ten peaks with the smallest $p$-value were selected, randomly combined, and fed into SVM. The accuracy of each combination in distinguishing PC from healthy control was analyzed; combinations with the highest accuracy were chosen as potential biomarkers, and the SVM model with the highest Youden index was used as the diagnostic model. The differential model of pattern 1 was comprised of three potential biomarkers, with m/z of 9,477, 7,411 and 14,804 Da. While the 9,477-Da peak had a higher expression level in PC model group, the other two peaks were higher expressed in healthy control group. The descriptive statistics of these three peaks are shown in Table I. Pattern 1 had 95.0% diagnostic specificity and 95.0% sensitivity, as evaluated by leave-one-out cross-validation (Table II).

PC model group versus gemcitabine-treated group (pattern 2). Pattern 2 was identified by comparing the peaks from mouse models of PC treated or not treated by gemcitabine. One hundred and eighty-eight qualified peaks were selected after noise filtering and peak cluster identification. These peaks were ranked according to the $p$-value of Wilcoxon rank sum test. The top ten peaks with the smallest $p$-value were selected, randomly combined, and fed into SVM. The accuracy of each combination in distinguishing PC from healthy control was analyzed; combinations with the highest accuracy were chosen as potential biomarkers, and the SVM model with the highest Youden index was used as the diagnostic model. The differential model of pattern 1 was comprised of three potential biomarkers, with m/z of 9,477, 7,411 and 14,804 Da. While the 9,477-Da peak had a higher expression level in PC model group, the other two peaks were higher expressed in healthy control group. The descriptive statistics of these three peaks are shown in Table I. Pattern 1 had 95.0% diagnostic specificity and 95.0% sensitivity, as evaluated by leave-one-out cross-validation (Table II).
noise filtering and peak cluster identification with the screening method described above. This differential model of pattern 2 was comprised of two potential biomarkers with m/z of 3,879 and 9,308 Da (Table I). The 3,879-Da peak had a higher level of expression in gemcitabine-treated group but a lower level in PC model group, while the 9,308-Da peak was lower in gemcitabine-treated group. This model had 95.0% specificity and 100.0% sensitivity, as evaluated by leave-one-out cross-validation (Table II).

**Pattern to distinguish healthy controls, PC model group and gemcitabine-treated group (pattern 3).** Aiming at optimizing the screening model of pattern 2, we added the healthy control group and formed pattern 3, which could better detect the alteration of protein peaks presenting in the whole process of chemoprevention model of PC. A total of 190 qualified peaks were initially selected and the final differential model of pattern 3 was comprised of three biomarkers with m/z of 9,308, 9,475 and 4,911 Da (Table III). Coincidently, the peak at 9,308 Da was also shown in pattern 2 (Fig. 3). Similarly, the peak at 9,308 Da was lower expressed in gemcitabine-treated group as compared to the PC model group, but highest expressed in healthy control group (Fig. 4). The peak at 9,475 Da initially weekly expressed in healthy controls, and then elevated when the PC model was established. After the treatment with gemcitabine, its expression was once again down regulated (higher than healthy control). The peak at 4,911 Da was also higher expressed in PC model group than in healthy control, but its expression level was even higher when treated with gemcitabine. As evaluated by leave-one-out cross-validation, the model had 95.0% specificity and 75.0% sensitivity (Table II), and the predicted accuracy of pattern 3 in healthy controls, PC model group and gemcitabine-treated group was 90.0, 85.0 and 95.0%, respectively.

**Discussion**

Previous studies have suggested combinations of serum biomarkers are more sensitive and specific than CA19-9 and are preferable in the diagnosis of PC (13-15). However, except for the CA19-9 level and imaging data, there is still lack of more accurate strategies which achieve to evaluate the prognosis of PC patients receiving chemotherapy or to assess the responsiveness of PC to chemotherapy. Aiming at detecting prognostic markers of PC after the responsiveness to chemotherapy, in this study, we used SELDI-TOF-MS, coupled with sophisticated bioinformatics approach, for complex data analysis, to disclose the serum protein ‘fingerprint’ of the chemoprevention model of PC. We identified for the first time three potential biomarkers to

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**Table I. Statistics of healthy controls versus PC model group (pattern 1) and PC model group versus gemcitabine-treated group (pattern 2).**

<table>
<thead>
<tr>
<th>m/z, Da</th>
<th>Healthy controls (A)</th>
<th>PC model group (B)</th>
<th>p-value</th>
<th>PC model group (B)</th>
<th>gemcitabine-treated group (C)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intensity in A</td>
<td>Intensity in B</td>
<td></td>
<td>Intensity in B</td>
<td>Intensity in C</td>
<td></td>
</tr>
<tr>
<td>9,477</td>
<td>528.03±143.65</td>
<td>5,744.01±8,997.26</td>
<td>9.27x10^-5</td>
<td>3879</td>
<td>1,610.02±700.89</td>
<td>9.27x10^-5</td>
</tr>
<tr>
<td>7,411</td>
<td>1,959.88±801.77</td>
<td>758.36±800.48</td>
<td>1.91x10^-4</td>
<td>9308</td>
<td>6,491.43±1,714.42</td>
<td>5.89x10^-4</td>
</tr>
<tr>
<td>14,804</td>
<td>2,970.64±1,292.15</td>
<td>1,039.79±1,227.75</td>
<td>5.25x10^-5</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table II. Predicted results of the differential pattern.**

<table>
<thead>
<tr>
<th>Pattern</th>
<th>Markers (n)</th>
<th>Training (%)</th>
<th>Leave-one-out cross-validation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3</td>
<td>100</td>
<td>95</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>95</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>95</td>
<td>85</td>
</tr>
</tbody>
</table>

**Table III. Statistics of peaks distinguish healthy controls, PC model group and gemcitabine-treated group (pattern 3).**

<table>
<thead>
<tr>
<th>m/z, Da</th>
<th>Intensity in healthy controls</th>
<th>Intensity in PC model group</th>
<th>Intensity in gemcitabine-treated group</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>9,308</td>
<td>8,196.47±1399.60</td>
<td>6,649.17±1,750.41</td>
<td>4,146.67±1,449.50</td>
<td>2.08x10^-6</td>
</tr>
<tr>
<td>9,475</td>
<td>495.89±146.54</td>
<td>5,535.95±8,653.74</td>
<td>4,284.80±4,505.47</td>
<td>8.91x10^-7</td>
</tr>
<tr>
<td>4,911</td>
<td>440.36±271.06</td>
<td>2,023.08±2,689.50</td>
<td>2,134.31±1,836.19</td>
<td>8.21x10^-6</td>
</tr>
</tbody>
</table>

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establish a protein ‘fingerprint’ model (pattern 3) to distinguish healthy controls, PC model group and gemcitabine-effective PC group at one time.

Analyzing the proteomic data of PC conducted by different research groups, it was found that the ‘fingerprint’ models or identified protein markers rarely overlapped with each other (13). Apart from the standardization and reproducibility of proteomics methodology, there are still several other reasons. Firstly, it is related to the complicated tissue compartments in PC. Proteins which form differentially expressed peaks could
derive from neoplastic cells, surrounding acini, as well as the surrounding stroma. A single model or identified protein can only highlight one or two features of PC. Secondly, inevitable potential sources of patient-related bias exist in clinical sampling, such as gender, age, genetics, environmental, dietary, psychological factors, previous therapy and various other factors, which could be crucial to the sample obtained and finally be the reason of deviation.

Well aware of its inadequacy, in this study, the combination of single diagnostic models (pattern 3) was established to minimize the heterogeneity of PC; and the orthotopic xenograft PC model on male BALB/c nu/nu mice was established to overcome the clinical patient-related bias. By utilizing the xenograft model, we could exclude the uncontrollable influential factors and focused on the biological nature of human PC. Besides, in this study, we optimized and standardized each step of the trial ranging from blood collection and clotting, to serum storage and handling, automated peptide extraction, crystallization, spectral acquisition and signal processing. With adequate technological and computational methods in place, and using rigorously standardized conditions, we found three differentially expressed protein peaks, which could distinguish healthy controls, PC model group and gemcitabine-treated group, with m/z of 9,308, 9,475, 4,911 Da. Among them, the peak at 9,308 Da overlapped the models (patterns 2 and 3). It could be considered as a potential chemoprotective protein marker in diagnosis and needs further investigation.

In this study, one of the challenges in data analyzing is to reduce the false protein peaks, in which the discriminatory power is due to random variation (17). To solve this problem, we developed ZUCI-PDAS, a bioinformatics tool, to analyze the spectral data. It included denoising with the UDWT, baseline correction, peaks detection, biomarker selection and evaluation of the SVM differential patterns. Our algorithm is likely to find most of the true, reproducible peaks. The SVM classification technique used in the ZUCI-PDAS is a new machine learning method based on the statistical theory (16). The SVM can solve problems such as the generalization of machine learning method based on the statistical theory (16). Liu Y: Active learning with support vector machine applied to tumor marker discovery. Ann NY Acad Sci 1022: 286-294, 2004.