Basic Research Paper

Dynamic changes of serum proteomic spectra in patients with non-Hodgkin lymphoma (NHL) before and after chemotherapy and screening of candidate biomarkers for NHL

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Background and Objective: Although the complete response rate of non-Hodgkin lymphoma (NHL) is 70–80% using modern comprehensive treatments, its relapse rate is about 40–50%. The minimal residual disease (MRD) may be the reason of recurrence. This study was to detect dynamic changes of serum proteomic spectra in NHL patients before and after chemotherapy, thus to screen candidate markers for NHL. Methods: The proteomic spectra from serum of 44 NHL patients, who achieved complete remission (CR), before and after chemotherapy, and 51 healthy individuals were analyzed by surface-enhanced laser desorption/ionization time of flight mass spectrometry (SELDI-TOF-MS) and Ciphergen ProteinChip 3.1 software. Results: Compared with the normal group, one protein peak (M11710) was up-regulated in untreated NHL group, while was close to the normal level in CR group (p < 0.05); nine other protein peaks (M3322, M4355, M6445, M6646, M8581, M8708, M8918, M13959, M15149) were down-regulated in untreated NHL group, while were close to normal levels in CR group (p < 0.05). Five candidate biomarkers for NHL were screened out using the decision tree model. Conclusions: Expressions of serum proteomic spectra are different before and after chemotherapy in NHL patients. Protein signatures of NHL may be screened using SELDI mass spectrometry combined with ProteinChip software. Those signatures may be helpful in screening MRD, detecting early recurrence and predicting the response to treatments.

Malignant lymphoma is a malignant disease occurring in the lymph nodes and/or lymph tissues beyond the lymph nodes. Although the complete remission (CR) rate for non-Hodgkin lymphoma (NHL) is 70–80%, 40–50% patients recurred and died at last. Recurrence of NHL is considered to be caused by minimal residual disease (MRD). Adopting NHL-specific serum proteins to monitor MRD and evaluate the treatment efficacy of NHL may help improve the life quality of NHL patients. MRDs in tumors have been investigated by researchers for decades. From the 1980s to 1990s, in vitro amplification techniques, such as PCR, Nested PCR, RT-PCR and the fluorescent quantitative PCR were applied to detect MRDs in NHL patients at the molecular level. However, no desirable results were obtained.1-4 Recently, the newly developed comparative proteomics is applied to investigate MRDs. This study applied surface-enhanced laser desorption/ionization time of flight mass spectrometry (SELDI-TOF-MS) technique to detect differential expression of serum proteomic spectra in NHL patients before and after chemotherapy, thus to explore the NHL-related or -specific biomarkers to monitor MRDs and predict the early recurrence of NHL.

Data and Methods

Source of samples. Forty-four NHL patients, who were initially treated in the Department of Internal Medicine in Sun Yat-sen University Cancer Center from February, 2003 to September, 2006 were enrolled. The patients included 35 males and nine females, ranged from seven months to 56 years, with a median age of 10 years. According to the pathological classification, 17 cases were T-cell lymphoblastic lymphoma/acute lymphoblastic leukemia (T-LBL/ALL), six cases were anaplastic large cell lymphoma (ALCL), one case was peripheral T-cell lymphoma (non-specific), eight cases were B-cell lymphoblastic lymphoma/leukemia (B-LBL/ALL), eight cases were diffuse large B-cell lymphoma (DLBCL), and four cases were Burkitt’s lymphoma (BL). Peripheral blood was extracted from all patients upon initial diagnosis and after achieving complete remission (CR) after the standardized chemotherapy. In total 88 blood samples were collected for spectral analysis. Serum samples from 51 healthy people, provided by the Sample Database in Sun Yat-sen University Cancer Center, were used as the normal control. A volume
of 4 ml peripheral blood was drawn and transferred into a dry test tube, centrifuged at 308 x g for 5 min, and stored in the fridge at -80°C.

**Materials.** The protein time-of-flight mass spectrometer (ProteinChip Biology System PBS II+) and the complementary weak cation exchanger protein chips (CM10) were purchased from Ciphergen Biosystem Company (USA). Urea, acetonitrile, trifluoroacetate, Cibacron Blue, Sinapinic acid (SPA) were purchased from Sigma Company (USA).

**Methods.** Preparation of serum samples. The blood samples were thawed in the icebox after being taken out from the fridge at -80°C, then centrifuged at 770 x g for 5 min. A volume of 10 μL serum was extracted and added to a 1.5 mL EP tube with 20 μL U9 buffer (9 mol/L urea plus 2% CHAPS). The samples were shaken in ice bath for 30 min at (30.8–46.2) x g.

**Chip balance.** The above prepared denaturalized sample (30 μL) was added into 360 μL CM10 buffer, and mixed rapidly to avoid sedimentation of proteins. The CM10 chip processor (Bioprocessor) was taken out carefully to avoid contact with the chip surface. The chip was then dipped into 200 μL CM10 buffer, shaken at (30.8–46.2) x g for 5 min in the oscillator to remove the buffer. The above procedure was repeated once.

**Combination of the chips and proteins.** When the buffer was shaken away twice, 100 μL processed samples were added into each well of the chip processor, shaken in the oscillator at (30.8–46.2) x g at room temperature for 1 h.

**Cleansing the chips.** When all the samples were shaken away, 200 μL CM10 buffer was added into each well of the chip processor, and shaken at (30.8–46.2) x g for 5 min. When the fluid was removed, 200 μL CM10 buffer was added and the procedure was repeated again.

**Addition of energy absorbing molecules (EAM).** A volume of 200 μL 1 mmol/L HEPES (pH 4.0) was added into each well of the chip processor. The fluid was shaken away at once. The chips were taken out of the chip processor. After the chips were air-dried, 0.5 μL SPA was added into each well. When the well was totally dried, 0.5 μL SPA was added again. When the samples were totally dried again, the chips were measured by the machine.

**Data reading.** All-in-one standard proteins were added into the chips. NP20 was used to adjust the SELDI mass spectrometer. When the parameters of the SELDI MASS spectrometer were set, the reading program in the Ciphergen ProteinChip software was adjusted. The protein combined with energy absorbing molecules (EAM) was detached from the chips under laser pulse excitation. In the electric field, proteins with different sizes flew at different speeds in the vacuum tube. The different flying time reflected the exact molecular weight (m/z) and the electron current of each protein. The values of m/z below 2000 were easily influenced by the matrix and other factors, and thus were eliminated. When the wave peaks were between 2000 and 20000, the Biomarker Wizard was used to filter the noises. The initial and second-time value of noise filtration were between 2000 and 20000, the Biomarker Wizard was used to filter the noises. The initial and second-time value of noise filtration were between 2000 and 20000, the Biomarker Wizard was used to filter the noises. The initial and second-time value of noise filtration were between 2000 and 20000, the Biomarker Wizard was used to filter the noises.
proteomic spectra were highly expressed in the NHL group compared to the normal group, seven of which did not show a decreasing tendency in the CR group after chemotherapy (p > 0.05). Six differential proteomic spectra were elevated in the NHL group compared to the CR group, and five of them were still overexpressed in the normal group (p > 0.05). M11710, which was negatively correlated with NHL, was screened out to be a small molecule biomarker for NHL. Recently, many researchers have been using the SELDI-TOF-MS proteomic spectra technique to identify tumor-specific biomarkers. Ye et al. found M11700, identified as the subtype of haptoglobin, was highly expressed in the ovarian cancer. Moshkovskii et al. revealed M11520 and M11681 in the ovarian cancer, which were identified as serum amyloid A1 (SAA-1) and its subtype. Le et al. detected M11488, M11573, M11639 and M11680 in prostate cancer, all of which were identified as SSA-1 and the subtype of SSA-1. Tolson et al. found that in renal cell carcinoma, M11144, M11423, M11518 and M11683 were highly expressed, and they were identified as SSA-1 and its subtypes. Cho et al. observed overexpression of M11600 and M11800 in the nasopharyngeal cancer, which were identified as SSA-1 and its subtype. However, the above-mentioned proteins detected by SELDI are acute-phase proteins, which are reactive products from the host, but not secreted by the cancer tissues. Though they are highly expressed in the serum of different cancer tissues, they are not tumor-specific. The biomarker M11710, which was discovered in the present study, needs to be isolated, identified and sequenced to determine its characteristics.

Our study only found one proteomic peak which is negatively correlated with NHL. This might be due to the following reasons: (1) the sera we used were obtained from patients with different subtypes of NHL, and the percentage of each subtype was low; (2) we only used CM10 chips, which might result in loss of much serum information. If various types of biological surface chips and chemical surface chips were applied, the whole serum map would have been obtained; and (3) low abundance proteins with plenty of information might not be detected. If the amount of the serum biomarker was too low, the marker protein could hardly successfully combine with the chips when they were competing with other irrelevant proteins.

Moreover, we found nine differential proteomic spectra which were negatively correlated with NHL, including M3322, M4355, M6445, M6646, M8581, M8708, M8918, M13959 and M15149. These proteomic spectra were lowly expressed in the NHL group compared with the normal group, and were increased to the normal level in the CR group. These proteins may be correlated to suppression of some proteins by some factors secreted by the tumors. The tumor abundance was reduced after chemotherapy, thus the levels of suppressed proteins return to normal again. These differential proteomic spectra are closely correlated with NHL, like M11710,
whose dynamical changes are corresponding to the occurrence and changes of tumor abundance. Identifications of these proteomic spectra need further investigation.

Because he nine proteins were rarely influenced by other factors, they were used to establish the decision tree model. M8581, M15149, M6646 and M8918 were found most closely related with NHL, and thus were identified as candidate biomarkers for NHL.

The NHL decision model showed a sensitivity rate between 93.3% and 100% when it was established. Half of the data were extracted from the model (in the NHL group) and the other half were from the CR group. The sensitivity rate decreased from 93.3% to 66.7% when it was used to screen for samples. The reasons that the accuracy rate was decreased when the model was used to classify the serum before and after chemotherapy are (1), the proteomic spectra used in the model might not be NHL specific biomarkers, and thus could not differentiate NHL serum from non-NHL serum completely; (2), CR was diagnosed based on imaging results or cyto-morphology, which could not exclude the influence of MRDs on serum proteomic spectra.

Five candidate biomarkers were found in NHL sera, which are M11710 (high expression), M8581 (low expression), M15149 (low expression), M6646 (low expression) and M8918 (low expression). The SELDI-TOF-MS proteomic chip techniques can be applied to screen the biomarkers in the NHL sera to provide useful information to monitor MRDs in NHL patients, predict the recurrence of early NHL, and evaluate the treatment efficacy.

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References