Detection of nasopharyngeal carcinoma using surface-enhanced laser desorption and ionization mass spectrometry profiles of the serum proteome

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[Abstract] Background and Objective: Early diagnosis of nasopharyngeal carcinoma (NPC) is difficult due to the insufficient specificity of the conventional examination method. This study was to investigate potential and consistent biomarkers for NPC, particularly for early detection of NPC. Methods: A proteomic pattern was identified in a training set (134 NPC patients and 73 control individuals) using the surface-enhanced laser desorption and ionization-mass spectrometry (SELDI-MS), and used to screen the test set (44 NPC patients and 25 control individuals) to determine the screening accuracy. To confirm the accuracy, it was used to test another group of 52 NPC patients and 32 healthy individuals at 6 months later. Results: Eight proteomic biomarkers with top-scored peak mass/charge ratios (m/z) of 8605 Da, 5320 Da, 5355 Da, 5380 Da, 5336 Da, 2791 Da, 7154 Da, and 9366 Da were selected as the potential markers of NPC with a sensitivity of 90.9% (40/44) and a specificity of 92.0% (23/25). The performance was better than the current diagnostic method by using the Epstein-Barr virus (EBV) capsid antigen IgA antibodies (VCA/IgA). Similar sensitivity (88.5%) and specificity (90.6%) were achieved in another group of 84 samples. Conclusion: SELDI-MS profiling might be a potential tool to identify patients with NPC, particularly at early clinical stages.

Key words: Nasopharyngeal neoplasms, mass spectrometry, surface-enhanced laser desorption/ionization, diagnosis, neural networks (computer), proteomic
sensitivity (~90%), yet its relatively low specificity (~80%) has limited its application. Therefore, a non-invasive detection method with both high sensitivity and specificity for screening NPC is necessary, especially for early diagnosis of NPC in the high incidence areas.

Due to the microheterogeneity of most human cancers, a combination of markers would be necessary for early detection of NPC. To achieve this, a rapid and high-throughput technology that can simultaneously analyze thousands of biomarkers is required. A recent advance in mass spectrometry (MS), the surface-enhanced laser desorption and ionization time-of-flight mass spectrometry (SELDI-TOF MS), is a sensitive system that is useful to generate proteomic patterns for cancer detection [7,8]. Its application has been proven convincing for early detection of ovarian, breast, prostate, and liver cancers [9-12] and for monitoring the prognosis of cancers [13,14].

This study aimed to determine whether SELDI profiling with weak cation exchange (WCX2) proteinchip array could be used to accurately screen out the NPC patients at an early stage.

Materials and Methods

Study subjects

NPC serum samples were obtained from 134 patients (101 men and 33 women) of 25–76 years old (median age 42) treated at Sun Yat-sen University Cancer Center between January 2000 and January 2001. Eight patients (6.0%) had differentiated non-keratinizing carcinoma (WHO type II), and 126 (94.0%) had undifferentiated non-keratinizing carcinoma (WHO type III). No patients received treatment before blood collection. According to Chinese 1992 TNM staging, 22 patients had stage I NPC, 33 stage II, 38 stage III, and 41 stage IV. Thirty-four healthy physical examiners (20 men and 14 women) aged 24–59 years (median age 39), working in Sun Yat-sen University Cancer Center, were selected as controls. Thirty-nine samples from NPC suspects (24 men and 15 women) aged 32–66 years (median age 42) were provided by the Department of Nasopharyngeal Carcinoma, Sun Yat-sen University Cancer Center. Of the 39 NPC suspects, 17 had residual nasopharyngeal adenoids, 13 had NPC first-degree relatives, and 9 had chronic nasopharyngitis. All of the patients with benign nasopharyngeal diseases had precluded the NPC diagnosis after 5-year follow-up visits. Patients with benign nasopharyngeal diseases were chosen as a part of control set because (1) they are more often to be suspected to have NPC and various diagnostic approaches are crucial for them; (2) this is the population in which the VCA/IgA screening marker shows false positive frequently; and (3) their serum samples, records of nasopharyngeal fiberscope-biopsy, and the 5-year follow-up information were available.

Serum samples from 52 patients with NPC and 32 healthy individuals were included for the subsequent testing. In NPC group, including 40 men and 12 women from 21 to 73 years old (median age 45), 4 patients (7.7%) had differentiated non-keratinizing NPC and 48 (92.7%) had undifferentiated non-keratinizing NPC. In control group, 32 healthy volunteers were from 26 to 61 years old (median age 38).

All serum samples were obtained in the early morning without food intake beforehand. They were then stored at −80°C until later use.

SELDI analysis

Serum samples were thawed and centrifuged at 594 × g for 5 min. Each sample (10 μL from each subject) was diluted in 90 μL solution containing 0.5% 3-[(3-chloroamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) (Sigma, USA) in phosphate buffer (pH 7.4), then added in 100 μL Cibacron Blue 3GA (Sigma, USA). The mixture was vortex-mixed at 4°C for 60 min and then centrifuged at 594 × g for 5 min. Collected supernatant (50 μL) was diluted in 150 μL of 100 mmol/L sodium acetate (pH 4.0) (Sigma, USA) and was added to bioprocessor (Ciphergen Biosystems, Fremont, CA), together with the WCX2 chips (Ciphergen Biosystems, Fremont, CA). The bioprocessor was then sealed and gently agitated for 60 min, then washed by 150 μL of 100 mmol/L sodium acetate thrice, 5 min each, and washed in deionized water twice, 1 min each. Chips were then removed from the bioprocessor and air-dried. Saturated solution (0.5 μL) of α-cyano-4-hydroxycinnamic acid (CHCA) (Sigma, USA), containing 50% acetoniitrile and 0.5% trifluoroacetic acid (Sigma, USA), was added to each chip array twice. The array surface was air-dried between the two CHCA applications.

The WCX2 proteinchip arrays were analyzed in a ProteinChip Software 3.1 Reader (PBS-II; Ciphergen Biosystems, Fremont, CA). The analytical parameters, high mass to 50 kDa, optimized from 1 kDa to 20 kDa, were applied for all the samples. The starting laser intensity was 160, with the starting detector sensitivity at 7. The result was obtained by averaging 91 laser shots of each sample. Peak detection was also performed using the Protein Software version 3.1 (Ciphergen Biosystems, CA).

To assess the precision and accuracy of the proteomic data, an all-in-one peptide molecular mass standard (Ciphergen Biosystems) was used as external calibration standard. The reproducibility of the protein chip was determined by using 6 serum samples from a single healthy individual. They were applied to the same batch or to
different batches of WCX2 chips and in-sample coefficient variance (CV) at different times were evaluated.

**Bioinformatics and biostatistics**

For the identification of NPC biomarkers, SELDI TOF MS was used to compare the samples from the patient and control groups. By the use of Ciphergen ProteinChip Software 3.1, all the spectra with a mass/charge ratio (m/z) between 1 and 50 kDa were filtrated twice with qualified signal-to-noise ratio. The peak clusters selected were simultaneously existed in at least 10% of the samples, with the same m/z within 0.3% mass window in different samples.

For each selected m/z peak, Mann-Whitney U test was performed and the difference in intensity was revealed. The m/z peaks with significant difference between the two groups were categorized and were characterized as the candidate biomarkers for NPC detection. A multi-layer perception (MLP) artificial neural network (ANN) with the scaled conjugate gradient (SCG) was adopted to optimize the back propagation algorithm in the discrimination of NPC samples from healthy individuals.

The classification model was trained by using different m/z peak subsets and by mean of further auto-optimizing, a combination of 8 peaks were selected to serve as final discriminating biomarkers. The sensitivity and specificity of the SELDI system was also calculated for the cancer and control samples in the blinded testing set.

Before analysis, all samples were randomly divided into training and test sets. One-third of the samples (n = 69) from 47 patients with NPC, 10 healthy volunteers, and 12 patients with benign nasopharyngeal diseases were selected as the blinded test set. The remaining 2/3 (n = 138) were selected as training set to define any discriminatory proteomic classifier with SELDI spectral and ANN analysis. We then used the classified pattern to predict the identity of other masked test samples.

Another group of 52 samples was analyzed by the SELDI system 6 months later to determine the accuracy of the selected candidate markers and their consistency of screening diseased samples.

All samples were analyzed with a VCA/IgA immunofluorescence kit (Sun Yan-sen Genetic Engineering Company, Guangzhou, China) according to the manufacturer’s instructions. A cut-off value of 1:10 titer was used as a positive criterion for the detection of VCA/IgA.

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**Results**

**Biomarker selection**

Our preliminary data showed that the WCX-2 surface chips provided the most promising spectra which allowed reliable discrimination between groups (data not shown). A set of 221 m/z peaks were selected as candidate biomarkers for distinguishing NPC and non-cancer individuals. Eight markers with top-scored m/z peaks (P < 0.000001) of 8605, 5320, 5355, 5380, 5336, 2791, 7154, and 9366 Da (Table 1, Figure 1) were selected for classification analyses and the accuracy (93.0%) achieved the highest (Figure 2). The markers with peaks of 8605, 2791, and 7154 Da were highly expressed in NPC group, whereas the others were highly expressed in the control group (Figure 3). Therefore, these markers were considered as potential biomarkers to screen NPC patients.

**Table 1** Details of the 8 potential biomarkers with top scored peaks for the detection of nasopharyngeal carcinoma

<table>
<thead>
<tr>
<th>m/z</th>
<th>Control</th>
<th>NPC</th>
</tr>
</thead>
<tbody>
<tr>
<td>8605</td>
<td>0.41 ± 0.20</td>
<td>0.73 ± 0.36</td>
</tr>
<tr>
<td>5320</td>
<td>1.68 ± 0.86</td>
<td>1.06 ± 0.50</td>
</tr>
<tr>
<td>5355</td>
<td>2.38 ± 0.95</td>
<td>1.60 ± 0.70</td>
</tr>
<tr>
<td>5380</td>
<td>1.46 ± 0.61</td>
<td>1.00 ± 0.45</td>
</tr>
<tr>
<td>5336</td>
<td>7.88 ± 4.43</td>
<td>4.96 ± 2.45</td>
</tr>
<tr>
<td>2791</td>
<td>0.65 ± 0.50</td>
<td>0.98 ± 0.62</td>
</tr>
<tr>
<td>7154</td>
<td>2.44 ± 1.33</td>
<td>4.08 ± 2.64</td>
</tr>
<tr>
<td>9366</td>
<td>1.26 ± 0.57</td>
<td>0.90 ± 0.26</td>
</tr>
</tbody>
</table>

m/z, mass/charge ratio; NPC, nasopharyngeal carcinoma. All values are presented as mean ± standard deviation. All P < 0.01.

**Evaluation of the classification model for NPC**

With the 8 selected peaks as input, the classification model was trained with 138 training samples, which then produced output values between 0 and 1. An output value less than 0.5 would predict the control group, whereas a value greater or equal to 0.5 would predict the patient group. Under this setting, the classification algorithm performed a 100% correctness in prediction of different samples. Analysis of the spectra from the 69 testing samples also revealed a high accuracy in identification (91.3%), with 90.9% for testing NPC samples and 92.0% for the control samples. For the subsequent testing carried out for another set of 52 samples 6 months later, similar sensitivity (88.5%) and specificity (90.6%) were obtained.

**Reproducibility and precision**

All-in-one peptide molecular mass standard allowed us to achieve a mass accuracy of about 1 Da in 10 kDa. With 6 samples from a single health individual, Figure 4 shows the representative examples of the six SELDI spectra within the region up to 80 kDa. The peaks are mostly seen for the...
Figure 1 Comparison of the 8 potential biomarkers with top scored peaks between nasopharyngeal carcinoma (NPC) and control groups

*Columns*, means of the height of the potential biomarkers; *bars*, standard errors. All $P < 0.01$.

Figure 2 The artificial neural network classification model was trained by using different m/z peak subsets before data collection

The eight top-scored peaks were summated to the model with the highest accuracy (0.9302) and minimal set of m/z peaks, and they were selected as the list of potential biomarkers.
Figure 3 The representative plots of the SELDI spectra and pseudo-gel views
The eight major subsets were compared between the NPC and the control groups. The arrows identify peaks with average m/z of 8605, 5320, 5355, 5380, 5336, 2791, 7154, and 9366 Da in NPC group compared with control group.
5 selected m/z peaks. After normalization, the highest amplitude was less than 15% and the CV for selected peaks mass was 0.05%.

**Serum VCA/IgA levels**

Serum VCA/IgA was titrated for all of the samples using immunoenzymatic method and a cut-off value of 1:10 was selected as a positive criterion. For the detection in NPC patients, the SELDI classification model, with a sensitivity of 86.4% (38/44), a specificity of 72.0% (18/25), and an accuracy of 81.2% (56/69), was undoubtedly better than conventional VCA/IgA assay. When we combined the 8 SELDI protein peaks and VCA/IgA as inputs to the integrated ANN, an improvement was evident in the accuracy in distinguishing NPC samples from controls. The sensitivity was promoted to 93.2%, the specificity was 92.0%, and the accuracy was 92.8%.

![Figure 4](image-url)

Figure 4 Reproducibility of representative SELDI-TOF MS from a single serum specimen processed on the same chip batch (lanes 1, 2 and 3) and on different batches (lanes 4, 5 and 6).

- After normalization, the highest amplitude was less than 15% and the CV for the five selected peaks mass (the arrows identified) was 0.05%.
- X axis: mass/charge ratio (m/z) values in Daltons; Y axis: relative intensity. Arrow near the peaks, the selected protein peaks.
Discussion

Taking advantage of the recent development in SELDI and ANN classification algorithm, we were able to simultaneously analyze the protein profiles of serum samples from patients with NPC, patients with benign nasopharyngeal diseases, and healthy individuals. This discriminate model led to the identification of several biomarkers that, in combination, achieved both high sensitivity and high specificity, which were higher than those of current most commonly used diagnostic marker VCA/IgA. Hence, the protein spectrum may be considered as a supplement to VCA/IgA.

Radiotherapy, the most common treatment for NPC, is usually sufficient to cure the patient with early stage NPC. It is cheaper than combination therapy, which is usually used for NPC at late stages. However, early detection of NPC is difficult due to different reasons: (1) the initial symptoms of early stage NPC such as sniffle, tinnitus, retropharyngeal bleeding, cervical lymphadenopathy are minimal and sometimes confusing to both the patients and the doctors; (2) the physical examination including the nasopharynx requires abundant clinical experience; (3) the growth of early stage tumor is very quick, which renders the disease into an advanced stage in a short period. Since NPC diagnosed at endemic regions are mostly in advanced stages, a reliable system which could provide early and accurate diagnosis would be beneficial to both the patients and the medical system.

NPC was found closely related to EBV, therefore, the EBV antigens with high antibody titers have been used as the diagnostic marker of NPC. Concurrently, there are also tests for detecting both immunoglobulin G (IgG) and immunoglobulin A (IgA), which respond to VCA, early antigen (EA), membrane antigen (MA), Epstein-Barr virus-coded nuclear antigen 2 (EBNA2), Epstein-Barr virus transactivator protein (ZEBRA), and Dnase. Another possible biomarker for NPC is EBV DNA in serum, however, the sensitivity and specificity of the assay are low. To date, VCA/IgA is still the most widely used biomarker for screening and early detection of NPC in Southern China. It is regarded as a sensitive and easy way to distinguish different clinical stages of NPC patients. Practically, it is a safe, highly compatible and repeatable test to use in clinics. However, this serological marker is not specific enough which give false positive results quite frequently, thus compromise the screening power of the assay. Furthermore, the difficulty in distinguishing false positive and VCA/IgA borderline results also renders the assay less reliable.

In the present study, we demonstrate that SELDI profiling of serum might be a superior method than the current standard serum biomarker VCA/IgA in distinguishing NPC. At approximately equal sensitivity (~90%), the specificity using SELDI classification model (92.0%) was significantly higher than that of conventional VCA/IgA (72.0%) (P < 0.001). The low molecular proteins found in the SELDI classification model are obviously different from the EBV antibody. To further improve the accuracy of NPC screening, the combined test of VCA/IgA and serum SELDI markers were carried out and showed a superior diagnostic performance versus any method used alone. Therefore, as a supplement for VCA/IgA assay, the serum SELDI markers would help to rule out the false-positive results in the conventional examination assay.

SELDI-TOF-MS technique is very sensitive and can detect 10–15 fmol proteins in serum. Therefore, only minor variation in the experimental procedures might largely affect the detection results. Since data of the same protein peaks could not be easily reproduced, standardization of laboratory procedures and tighten the quality control become necessary. In our study, all serum samples were obtained in early morning and stored in the same temperature.

Experimental procedures were strictly followed. In addition, all-in-one peptide molecular mass standard was used to control a mass accuracy of about 1 Da in 10 kDa. In order to test the reproducibility of MS, 6 serum samples from 1 individual were detected to different batches of WCX2 chips. The CV for the selected peaks was 0.05% and was consistent with the reproducibility data for the PBSIITOF MS reported by the manufacturer (Ciphergen Biosystems, Fremont CA). Furthermore, similar sensitivity and specificity were obtained for another NPC and control samples after the primary SELDI detection using the same classification model for NPC. These results indicate a promising reproducibility of the discriminating protein profiles for NPC.

To optimize the combination of serum markers for cancer detection, sophisticated bioinformatic tools for complex data analysis and pattern recognition are required for high-throughput proteomic profiling. In the present study, a powerful ANN genetic algorithm and multi-layer perception ANN were used for the analysis of SELDI-MS data. This type of ANN is suitable to analyze complex data with high level of background noise, which can also be used to identify the influence of interacting factors, including the SELDI-MS data in the present study. According to previous study, ANNs can generalize models with a greater accuracy without relying on predetermined relationships as in other modeling techniques, for medical diagnostic purpose.

In conclusion, the parallel use of SELDI-TOF MS and VCA/IgA assay might provide a powerful tool for NPC screening with high accuracy, sensitivity and specificity.
Chinese Journal of Cancer

References