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# Identifying FGA Peptides as Nasopharyngeal Carcinoma-Associated Biomarkers by Magnetic Beads

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## ABSTRACT

Early diagnosis and treatment is known to improve prognosis for nasopharyngeal carcinoma (NPC). The study determined the specific peptide profiles by comparing the serum differences between NPC patients and healthy controls, and provided the basis for the diagnostic model and identification of specific biomarkers of NPC. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) can be used to detect the molecular mass of peptides. Mass spectra of peptides were generated after extracting and purification of 40 NPC samples in the training set, 21 in the single center validation set and 99 in the multicenter validation set using weak cationic-exchanger magnetic beads. The spectra were analyzed statistically using FlexAnalysis™ and ClinProt™ bioinformatics software. The four most significant peaks were selected out to train a genetic algorithm model to diagnose NPC. The diagnostic sensitivity and specificity were 100% and 100% in the training set, 90.5% and 88.9% in the single center validation set, 91.9% and 83.3% in the multicenter validation set, and the false positive rate (FPR) and false negative rate (FNR) were obviously lower in the NPC group (FPR, 16.7%; FNR, 8.1%) than in the other cancer group (FPR, 39%; FNR, 61%), respectively. So, the diagnostic model including four peptides can be suitable for NPC but not for other cancers. FGA peptide fragments identified may serve as tumor-associated biomarkers for NPC. J. Cell. Biochem. 113: 2268–2278, 2012. 

Abbreviations: NPC, nasopharyngeal carcinoma; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; WCX-MB, weak cationic-exchanger magnetic bead; CV, coefficient of variation; BS, binding solution; WS, washing solution; ES, eluting solution; SS, stabilizing solution; nano-LC/ESI–MS/MS, nano-liquid chromatography–electrospray ionization–tandem mass spectrometry; GA, genetic algorithm; PPV, positive predictive value; NPV, negative predictive value; FPR, false positive rate; FNR, false negative rate; FGA, fibrinogen alpha chain; Fg, fibrinogen; TIC, total ion current; MS, mass spectrometry; MS/MS, tandem mass spectrometry; m/z, mass-to-charge ratio.

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**KEY WORDS:** SERUM BIOMARKER; NASOPHARYNGEAL CARCINOMA; FGA; MAGNETIC BEAD

Nasopharyngeal carcinoma (NPC) is one of the most common cancer in China, especially in Southern China where the incidence rate is approximately 30–80/100,000 [Chen et al., 2008] people per year. Radiotherapy is still the most main therapy of comprehensive treatment for NPC [Lo et al., 2000; Zou et al., 2000]. The 5 years of survival rate of the early NPC is 90%; the advanced NPC is 70% [Lee et al., 2005; Chen et al., 2008; Gao et al., 2010]. Therefore, the survival rate of NPC can be dramatically improved through the early diagnosis.

Serum contains numerous secreted or shed low abundance proteins that are critical for signaling cascades and regulatory events. Cancer may be accompanied by the production and release into the blood of a substantial number of proteins and/or hormones that could serve as useful markers for assessing prognosis, monitoring treatment, and detecting malignant disease at an early stage. Methods for parallel analysis of the expression of a large number of protein in the blood are evolving rapidly. The classical approach consists of three major techniques: two dimensional electrophoresis (2-DE), surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS) [Xiao et al., 2001; Wellmann et al., 2002; Shiwa et al., 2003; Zheng et al., 2003] and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). Indeed, proteomic method based on beads fractionation and MALDI-TOF-MS analysis can sensitively and precisely separates target proteins according to their mass-dependent velocities (m/z), which has recently been successfully applied to the diagnosis of cancer [Seliger and Kellner, 2002], such as prostate cancer [Villanueva et al., 2006b], oral cancer [Cheng et al., 2005], thyroid cancer [Villanueva et al., 2006a], and hepatocellular carcinoma.

Recently, we have developed affinity bead-based purification technique which can reduce cost and make proteomic procedures suitable for general MS analysis [Tao et al., 2011]. This approach is sensitive and fast, features essential for clinical use, whereas the limitations of SELDI analysis include high cost, the fact that only one mass spectrometry pass is made, and difficulty in further protein identification. Using this method to detect a small sample set, we initially established the diagnostic model of NPC, whose accuracy rate was about 80%. More research is still required before final goal of increasing diagnostic efficacy and identifying the specific biomarkers of NPC. In our study, we chose the weak cationic-exchanger magnetic bead (WCX-MB) to purify peptides in the serum, used MALDI-TOF MS to obtain peptide expression profiles from the serum samples with and without NPC, established and tested the diagnostic classification through a genetic algorithm (GA) [Chen et al., 2004; Jeffries, 2004] and finally identified the serum biomarkers for NPC.

## MATERIALS AND METHODS

### EXPERIMENTAL DESIGN AND FLOWCHART

This study consisted of three parts: first discovery screen for peptide differences by serum proteomics based on the magnetic beads was done, second a GA model to diagnose NPC was established in the training set and validated in the single center and multicenter independent testing sets, and third the sequences of the most promising protein/peptide biomarkers of NPC were identified with LTQ-Orbitrap-MS. The flowchart of the study was summarized in the Figure 1. In the first part of peptide differences screen, a comparative proteome analysis was carried out to identify a series of peptides which could be used to diagnose NPC, a p-value filter strategy was used to select and optimize peptide peaks with statistically significant differences between the NPC and the healthy control. In the model established part, the serum peptide profiles in the training set were analyzed using ClinProt$^{TM}$ software v.2.1 with the GA. The accuracy of the predictive model for NPC was subsequently validated in the single center and multicenter independent testing set, respectively. At last, the most promising protein/peptide biomarkers were identified for the diagnosis of NPC patients.

### SAMPLES GROUPING AND PREPARATION

This study was approved by the ethics committee of Cancer Center, Sun Yat-sen University. Patients in the study were pathologically diagnosed as NPC and were treated according to treatment guideline. All the samples were collected before radiotherapy at seven hospitals (Sun Yat-Sen University Cancer Center, The First Affiliated Hospital of Guangzhou Medical University, General Hospital of Guangzhou Military Command of PLA, The First Affiliated Hospital of Jinan University, The Fifth Affiliated Hospital of Sun Yat-Sen University, Huizhou Municipal Central Hospital and Dongguan People's Hospital). The clinical characteristics of NPC patients and healthy controls were shown in Table I. As shown in Figure 1, all the samples were divided into two sets: training set and validation set. According to the source of samples, the validation set included the single center set and the multicenter set. In addition, we add the other cancer group to evaluate whether the model was specific for NPC or not.

The serum samples were prepared according to standard protocol. Fasting blood samples were collected from patients in the morning and allowed to clot at 37°C for 0.5 h; sera were then separated by centrifugation at 2,000 rpm for 15 min and stored at −80°C until analysis. For the reproducibility experiments, sera from 10 patients with NPC were pooled and the same MALDI-TOF-MS instrument was then used to run six within-run assays and six between-run assays to see the deviation. Coefficient of variation (CV) was calculated. The mean CV of within-run assays was 15.21% (12.0–20.9%) and that of between-run assays was 15.50% (8.6–22.9%).

### PROTEOMICS ANALYSIS FOR THE SERUM PEPTIDES DIFFERENCES

**Serum pretreatment with magnetic beads.** All the serum samples were fractionated using weak cation exchange magnetic beads (WCX-MB) according to the manufacturers' instructions through a standard protocol (ClinProt$^{TM}$, Bruker Daltonics). Samples were purified and isolated through three steps: binding, washing, and elution. Firstly, 10 μl beads, 10 μl WCX-MB binding solution (BS) and 5 μl serum sample were added in a tube and mixed carefully and

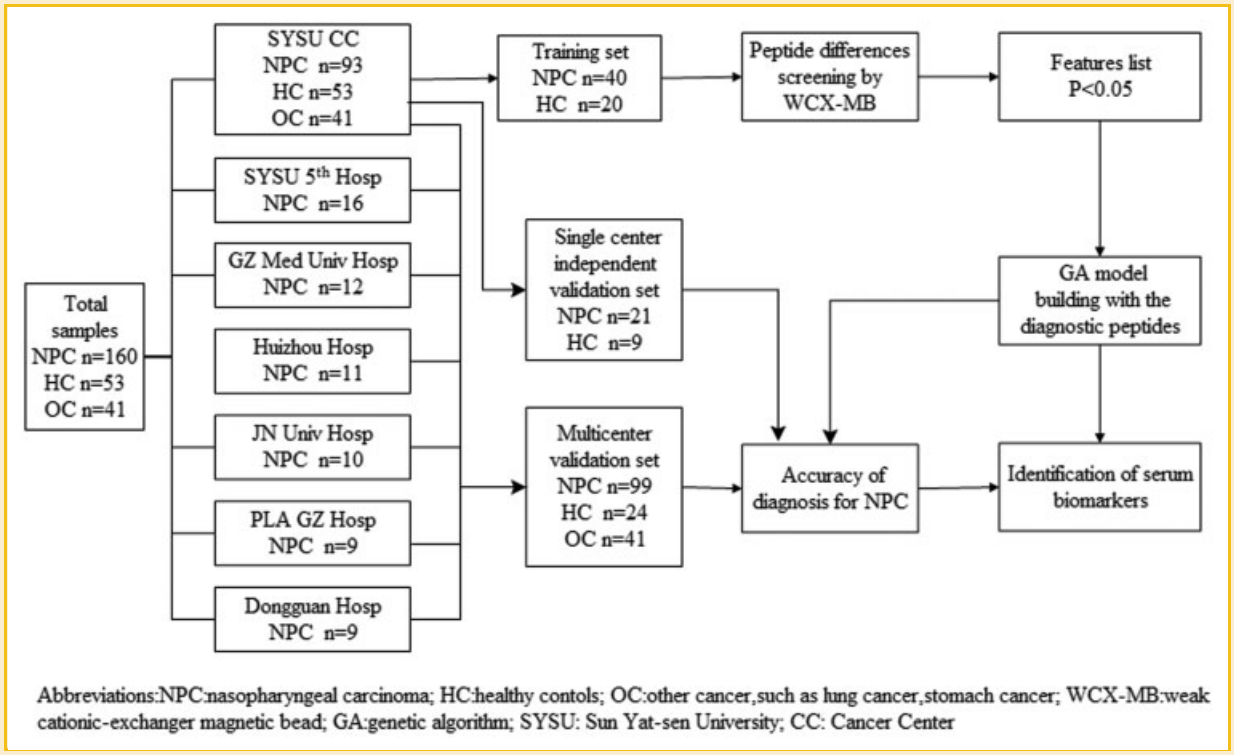


Fig. 1. Study flowchart.

incubated for 5 min. Place the tube on the magnetic bead separation device (Bruker Daltonics) and collect the beads at the tube wall for 1 min. Then, remove the supernatant and add 100 μl magnetic bead washing solution (WS), mix them thoroughly. After washing for three times, remove the supernatant, add another 5 μl magnetic bead eluting solution (ES), and collect the beads at the tube wall in the separation device for 2 min. Finally, transfer the clear supernatant into a fresh tube, add 5 μl magnetic bead stabilizing solution (SS) to the eluate and mix them intensively, store the sample tube in refrigerator (−20°C).

**Anchor chip spotting and protein/peptide profiling.** The eluted sample was diluted 1:10 in matrix solution α-cyano-4-hydroxycinnamic acid (0.3 g/L in ethanol: acetone 2:1), which was daily prepared. For example, 1 μl of eluate was added to 10 μl matrix solution. Then 1 μl of the mixture was spot onto a MALDI-TOF-MS target (AnchorChip™, Bruker Daltonics) and dried at room temperature before analysis. MALDI-TOF-MS measurements were performed using an Autoflex TOF instrument (Bruker Daltonics). Before MS analysis, it was important to have the system quality controlled. Eleven peptides were used as external standard preparation and the average molecular weight deviation was no more than 100 μg/g. Before data acquisition of every eight samples, the standard preparation would be calibrated. Additionally, 13 reference sera were added as external standard too. The coefficient of variability <30% indicated that the system ran well. Profile spectra were acquired from an average of 400 laser shots per sample. Determine the peak m/z values or intensities in the mass range of 1,000–10,000 Da.

## DIAGNOSTIC MODEL FOR NPC WITH THE GA CLASSIFIER

**Establishment of model in the training set.** The serum peptide profiles of 40 patients with NPC and 20 healthy controls in the training set were analyzed. The reproducibility of mass spectral generation was determined by the mean relative peak intensities. All the spectra obtained from the serum samples in the training set were analyzed using ClinProTools (ClinProt software version 2.0; Bruker Daltonics) to subtract baseline, normalize spectra (using total ion current) and determine peak m/z values and intensities in the mass range of 1,000–10,000 Da. The signal-to-noise (S/N) ratio should be higher than five. To align the spectra, a mass shift of no more than 0.1% was determined. The peak area was used as quantitative standardization. GA contained in this software suite was used to establish the best pattern for identifying NPC. After each profile was generated, a 20% leave out cross-validation process was performed within the software. Comparison of relative peak intensity levels between two proups was also calculated within the software suite. Student's *t*-test was used for analysis of normally distributed continuous data, while Wilcoxon test for non-normally distributed continuous data. Chi-square test was used for categorical data analysis. $P < 0.05$ was considered statistically significant.

**Validation for model in the independent testing set.** The accuracy of NPC diagnosis was validated in the single center and multicenter independent testing set. All the spectra in the testing set was followed the same steps mentioned before. Thus, the sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and the total accuracy of NPC model were calculated.

TABLE I. Clinical Characteristics of NPC Patients and Controls

| Variable | No. of patients (%) | | | | | | |
|---|---|---|---|---|---|---|---|
| | Training set (n = 60) | | Single center independent validation set, (n = 71) | | | Multi center independent validation set (n = 123) | |
| | NPC patients (n = 40) | Healthy controls (n = 20) | NPC patients (n = 21) | Healthy controls (n = 9) | Other cancer patients (n = 41) | NPC patients (n = 99) | Healthy controls (n = 24) |
| Mean age ± SD | 43.6 ± 12.1 | 32.2 ± 5.8 | 46.5 ± 10.1 | 29.3 ± 7.5 | 52.7 ± 10.9 | 45.9 ± 11.2 | 31.5 ± 5.9 |
| Gender | | | | | | | |
| Male | 25 (62.5) | 13 (65) | 19 (90.5) | 4 (44.4) | 18 (43.9) | 72 (72.7) | 15 (62.5) |
| Female | 15 (37.5) | 7 (35) | 2 (9.532) | 5 (55.6) | 23 (56.1) | 27 (27.3) | 9 (37.5) |
| Tumor | | | | | | | |
| Gastric carcinoma | | | | | 4 (9.8) | | |
| Esophageal carcinoma | | | | | 1 (2.5) | | |
| Cervical carcinoma | | | | | 6 (14.6) | | |
| HCC | | | | | 5 (12.2) | | |
| CRC | | | | | 8 (19.5) | | |
| Lung cancer | | | | | 8 (19.5) | | |
| Breast cancer | | | | | 6 (14.6) | | |
| Thyroid carcinoma | | | | | 3 (7.3) | | |

HCC, hepatocellular carcinoma; CRC, colorectal carcinoma.

In addition, the false positive rate (FPR) and false negative rate (FNR) were calculated to evaluate the specificity of the GA model in the other cancer validation set.

### IDENTIFICATION OF PROTEIN BIOMARKER BY NANO-LC/ESI–MS/MS

**Peptide sequence.** The sequences of differential expression peptides between NPC and control were identify using a nano-liquid chromatography–electrospray ionization–tandem mass spectrometry (nano-LC/ESI–MS/MS) system consisting of anAquity UPLC system (Waters) and a LTQ Obitrap XL mass spectrometer (Thermo Fisher) equipped with a nano-ESI source. MS/MS experimental protocol was as follow. The peptide solutions were loaded to a C18 trap column (nanoACQUITY) [180 μm × 20 mm × 5 μm (symmetry)] with the flow rate of 15 μl/min. Then, the desalted peptides were enriched by C18 analytical column (nanoACQUITY) [75μm × 150 mm × 3.5μm (symmetry)] at a flow rate of 400 μl/min. Thirdly, the mobile phases A (5% acetonitrile, 0.1% formic acid) and B (95% acetonitrile, 0.1% formic acid) were used for analytical columns. Peptides were eluted using solvent B to the gradient elution profile, such as 5%B–50%B–80%B–80%B–50%B–5%B in 100 min. The MS instrument was operated in a data-dependent model. The range of full scan was 400–10,000 m/z. The eight most intense monoisotope ions were the precursors for collision induced dissociation. MS/MS spectra were limited to two consecutive scans per precursor ion followed by 60 s of dynamic exclusion.

**Bioinformatics and identification of serum biomarkers.** The obtained chromatograms were analyzed with BioworksBrowser 3.3.1 SP1 and the resulting mass lists were used for database search using Sequest™ [IPI Human (3.45)]. Parameters for generating peak list were as follows: parent ion and fragment mass relative accuracy were set at 50 μg/g and 1 Da, respectively.

## RESULTS

### REPRODUCIBILITY AND PRECISION

To assess the precision and accuracy of the proteomic data in our analyses, sera from 10 patients with NPC were pooled and the same MALDI-TOF-MS instrument was then used to run six within-run assays and six between-run assays to see the deviation. CV was calculated and shown in Table II. The mean CV of within-run assays was 15.21% (12.0–20.9%) and that of between-run assays was 15.50% (8.6–22.9%).

### DISCOVERY SCREENING OF PEPTIDE DIFFERENCES

To screen serum peptides of interest for NPC diagnosis, 40 patients with NPC and 20 healthy control serum samples in training set were analyzed by MALDI-TOF-MS with WCX-MB. A total of 95 peaks were detected from m/z spectra ranging from 1,000 to 10,000 Da in the training set. We used two-tailed *t*-test to obtain a *P*-value for each peak and rank the peaks with *P*-value. Using $P < 0.05$, $P < 0.001$, and $P < 0.0001$ cutoffs, 43, 25, and 10 features were generated out of the total 95 peaks, respectively (shown in Table III).

TABLE II. Reproducibility of Mass Spectra Profiled by Magnetic Beads and MALDI-TOF-MS Analysis

| Signal m/z | Whithin-run | | Between-run | |
|---|---|---|---|---|
| | MRI (%) | CV (%) | MRI (%) | CV (%) |
| 1,207 | 10.3 | 16.3 | 10.1 | 8.6 |
| 1,351 | 3.7 | 12.0 | 4.5 | 16.2 |
| 2,554 | 3.5 | 20.9 | 10.0 | 15.7 |
| 6,635 | 22.0 | 14.8 | 12.9 | 22.9 |
| 1,207 | 12.2 | 13.6 | 12.5 | 17.9 |
| 1,351 | 5.8 | 15.1 | 7.7 | 16.4 |
| 3,315 | 3.6 | 13.8 | 2.7 | 10.8 |

m/z, average mass; MRI, mean relative intensity; CV, individual coefficient of variation.

### ASSESSMENT OF DIAGNOSTIC EFFICACY

**Establishing model in the training set.** GA was utilized to generate models to discriminate NPC patients from the healthy controls in training set using features with $P < 0.05$. Twenty percent of the recruited subjects were assigned randomly to establish model and the rest were used for cross-validation. As shown in Figure 2, serum peptide patterns in NPC samples differed significantly from ones in the healthy controls.

The peaks of all the serum profiles in the training set were ranked by a *t*-test with ClinProt™ software v.2.1 to rank their discriminatory power between NPCs and controls. As shown in Figure 3, A was feature performance of m/z 1262.67 and m/z 3193.14 in the receiver operating characteristic (ROC) curve, respectively. When the peptide with m/z 1262.67 was used to distinguish NPC patients from healthy controls, the area under the curve (AUC) of ROC curve was 0.83. The AUC of m/z 3193.14 was 0.84. B was clustering analysis of 23 peaks with significant difference ($P < 0.001$, by *t*-test) in their distribution between NPC and control samples. The intensities of the 23 peaks in 60 samples in binary format was arranged by unsupervised, average-linkage hierarchical clustering between NPC patients and the healthy controls. Columns represented samples; rows were m/z peaks as indicated by the average molecular weight. With the increase of the remission degree, in the NPC samples, the peptides relative intensities with m/z 1262.67 was found to be gradually increased, and ones with m/z 3193.14 gradually decreased (Fig. 4).

**Validating model in the testing set.** As indicated in the Table IV, a GA model established with spectra of 3193.14 m/z, 1051.37 m/z, 4055.47 m/z, and 1262.67 m/z in the training set correctly classified all of the 40 NPC samples as positive, and all the 20 healthy samples as negative, yielding both 100% sensitivity and 100% specificity. The accuracy of diagnostic prediction was validated in the single center and multicenter independent testing set with 120 NPC patients and 33 healthy samples. Blind evaluation was as follow: the sensitivity was 90.5%, the specificity 88.9%, the PPV 95%, the NPV 80% and the accuracy 90% in the single center set, and the sensitivity 91.9%, the specificity 83.3%, the PPV 95.9% and the NPV 71.4% and the accuracy 90.2% in the multicenter set (shown in Table IV). In the other cancers group, the GA model of NPC classified 16 of 41 samples as positive and the other 25 as negative in the testing set, yielding 39% FPR and 61% FNR which were higher than that in the NPC group (FPR, 16.7%; FNR, 8.1%), respectively.

TABLE III. Distribution of *P*-Value Markers Between NPC Group and Controls

| m/z | MRI (SD) in NPC | MRI (SD) in controls | *P*-value |
|---|---|---|---|
| 1089.68 | 80.95 (55.25) | 30.19 (12.77) | 7.12E−05 |
| 4055.47 | 36.7 (18.52) | 77.03 (39.2) | 7.12E−05 |
| 3193.14 | 24.53 (16.31) | 58.37 (36.56) | 7.12E−05 |
| 1051.37 | 45.3 (34.6) | 12.08 (4.75) | 7.12E−05 |
| 1300.51 | 52.94 (31.98) | 20.92 (7.05) | 7.12E−05 |
| 1290.91 | 40.9 (25.73) | 14.38 (5.69) | 7.12E−05 |
| 1034.84 | 32.8 (17.73) | 13.78 (4.37) | 7.12E−05 |
| 1009.03 | 26.64 (13.69) | 11.99 (3.6) | 7.12E−05 |
| 911.05 | 9.59 (7.6) | 2.44 (1.14) | 7.12E−05 |
| 992.26 | 13.49 (5.84) | 6.99 (2.64) | 7.12E−05 |
| 1262.67 | 31.4 (21.19) | 10.21 (3.35) | 0.000101 |
| 882.83 | 120.34 (93.13) | 35.45 (22.19) | 0.00011 |
| 1073.29 | 180.02 (120.06) | 66.61 (34.26) | 0.000134 |
| 904.45 | 37.14 (26.08) | 11.92 (5.07) | 0.000134 |
| 1503.81 | 21.7 (12.07) | 9.71 (3.71) | 0.000134 |
| 1279.41 | 88.23 (59.11) | 31.36 (15.16) | 0.000152 |
| 1489.85 | 42.61 (23.38) | 19.01 (7.67) | 0.000152 |
| 917.87 | 26.35 (22.09) | 10.96 (18.99) | 0.000156 |
| 3261.35 | 56.4 (43.33) | 127.45 (74.73) | 0.000174 |
| 2023.28 | 22.66 (9.35) | 51.35 (30.72) | 0.000194 |
| 4073.91 | 24.81 (10.22) | 41.62 (17.49) | 0.000216 |
| 3176.27 | 17.43 (7.82) | 28.9 (12.54) | 0.000223 |
| 898.91 | 9.54 (7.81) | 2.6 (1.57) | 0.000658 |
| 3241.84 | 22.66 (22.29) | 60.96 (49.62) | 0.000925 |
| 3228.62 | 6.17 (3.3) | 13.08 (9.13) | 0.000925 |
| 2082.76 | 16.4 (7.69) | 37.49 (32.91) | 0.00109 |
| 1897.67 | 16.75 (5.96) | 33.82 (25.93) | 0.00109 |
| 3144.86 | 13.87 (4.95) | 21.07 (8.39) | 0.00109 |
| 1885.44 | 17.69 (7.27) | 30.45 (14.57) | 0.00112 |
| 1866.37 | 16.97 (14.03) | 53.85 (49.71) | 0.00223 |
| 2660.78 | 33.04 (23.71) | 66.29 (61.29) | 0.00376 |
| 4092.2 | 52.47 (23.12) | 75.88 (32.64) | 0.00691 |
| 4301.95 | 30.14 (20.91) | 25.02 (40.04) | 0.00708 |
| 3208.98 | 41.78 (25.17) | 66.18 (42.16) | 0.00727 |
| 4211.24 | 244.79 (121.32) | 352.71 (148.36) | 0.00881 |
| 1531.34 | 21.17 (10.64) | 13.24 (4.79) | 0.0106 |
| 1946.58 | 113.06 (207.68) | 190.6 (184.92) | 0.0141 |
| 4111.08 | 22.78 (8.11) | 31.71 (13.5) | 0.0145 |
| 1330.17 | 25.64 (14.44) | 15.8 (4.22) | 0.0173 |
| 1117.83 | 29.64 (22.28) | 15.15 (5.28) | 0.0177 |
| 2883.62 | 35.66 (22.06) | 50.95 (30.8) | 0.022 |
| 5340.01 | 54.77 (55.03) | 79.42 (54.63) | 0.0419 |
| 4230.02 | 49 (24.69) | 65.64 (33.17) | 0.0419 |

MRI, mean relative intensity; SD, standard deviation.

### IDENTIFICATION OF THE SERUM BIOMARKERS FOR NPC

With LTQ-Orbitrap-MS detection, 30 of 43 peptides (showed in the Table III) of differential expression of between NPC and healthy controls were identified successfully, and the others were unsuccessful (showed in theTable V). The sequences of four diagnostic peptides, whose molecular weight were, respectively, 3193.14 m/z, 1051.37 m/z, 4055.47 m/z, and 1262.67 m/z, used to establish the GA model were identified as FGA isoform 1 of Fibrinogen alpha chain precursor (highlighted in bold, Table V). A representative sequencing result of m/z 1263.67 was shown in Figure 5.

## DISCUSSION

Serological test has many advantages as below. (i) Samples are easily collected. (ii) Non-invasive testing can greatly reduce the examinational pain of patients. (iii) It is available for tumors which are hard to obtain tissue specimens. Thus, many researchers hypothesized that the best cancer biomarkers will likely be proteins

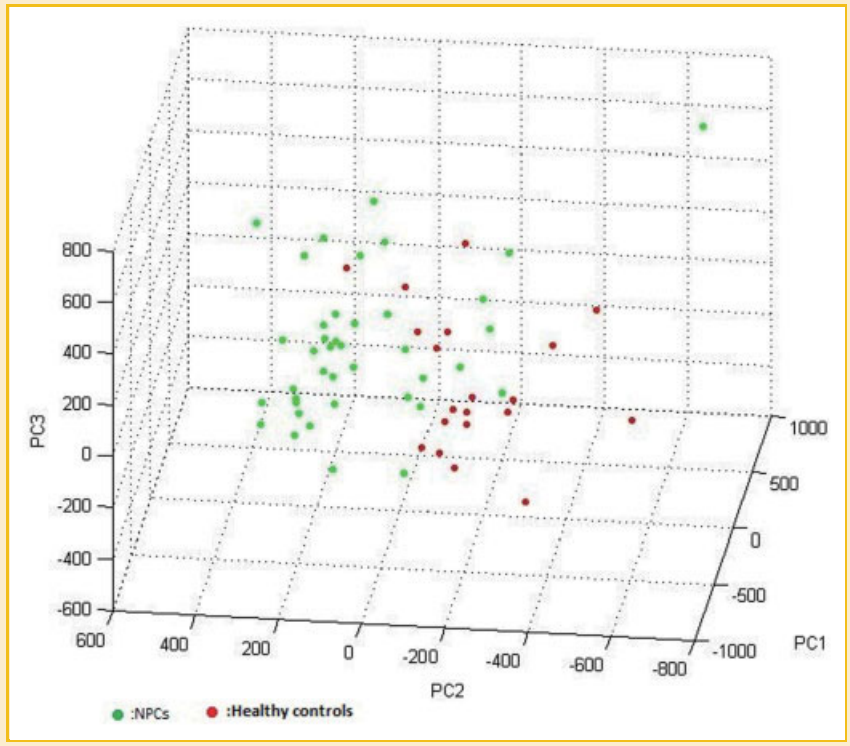


Fig. 2. Principal component analysis of MS-based serum peptide profiling data derived from NPC and healthy controls. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

secreted in blood [Welsh et al., 2003]. Proteomic analyses based on mass spectrometry technique provided us the innovative ways to identify the components of protein complexes in serum. Petricoin et al. [2002] have pioneered the use of mass spectrometry as a diagnostic tool. A simple straightforward serum procedure for all the high risk people would be an ideal diagnostic tool to predict the early carcinoma. Most studies have been carried out by SELDI-TOF technology in different types of cancers, including ovarian cancer, prostate cancer, breast cancer, and bladder cancer, and achieve very high diagnostic sensitivity and specificity (approaching 100%) in comparison to the classical cancer biomarkers [Issaq et al., 2003; Powell, 2003]. Especially for NPC, Ho et al. [2006] established the diagnostic model with six potential novel NPC biomarkers [6,692, 6,811, 6,862 Da, 7,979, 9,176, and 10,272 Da) The sensitivity and specificity were 80% and 84%, respectively. Wei et al. chose 4 protein peaks (4,097, 4,180, 5,912, and 8,295 Da) as a biomarker pattern whose sensitivity and specificity were 92% and 92.9% [Wei et al., 2008]. A panel of three biomarkers (3159.83, 5187.65, and 13738.6 Da) was selected to establish Decision Tree model with sensitivity of 95.0% and specificity of 83.33% in Huang et al.'s study [2009]. However, the SELDI analysis has the limitations including high cost, the fact that only one mass spectrometry pass is made, and difficulty in further protein identification.

So in this study, we directly profiled protein/peptide patterns from affinity bead-purified serum samples with MALDI-TOF MS and established a model including four serum peptide biomarkers through GA that discriminated NPCs from healthy controls. The specific procedure was as follows. Firstly, NPC and healthy control serum samples were detected by MALDI TOF MS and peptide peaks of all the samples were obtained. Secondly, two-tailed *t*-test was used to compare intensity of peptide peaks between NPC group and control group and the peptide peak with *P*-value of $<0.05$ was considered as significant differential peaks (shown in Table III). Thirdly, the model for diagnosis of NPC was established from significant differential peaks and its statistical method was as follows: (i) K-nearest neighbor (K-NN) algorithms, GA, and neural network algorithm were used to create several candidate models for diagnosis of NPC. (ii) The classification error rate in the cross-validation of the training set was used to select the best model, the classification error rate of which was lowest. (iii) The accuracy, sensitivity, specificity, PPV, and NPV of model were calculated, then the model were validated in the independent testing set. Finally, we found a combined markers (3193.14 m/z, 1051.37 m/z, 4055.47 m/z, 1262.67 m/z) that provided high sensitivity (100%) and specificity (100%), and the use of these combined markers as a significant discriminator was validated in the multicenter independent testing set yielding the sensitivity of 91.9%, the specificity of 83.3% and accuracy of 90.2%. The FPR and FNR were higher in the other cancer group (FPR, 39%; FNR, 61%) than in the NPC group (FPR, 16.7%; FNR, 8.1%), respectively. The results indicated that the NPC model was more suitable for the diagnosis of NPC than other cancers.

Up to now, there were many studies to discover the biomarkers for NPC, which were summarized according to their function as follows [Li et al., 2008; Feng et al., 2010; Cai et al., 2011; Chang et al., 2011; Tai et al., 2012; Wu et al., 2012; Zeng et al., 2011]. (i) Biomarkers for diagnosis. Analyzing secreted-proteome of two NPC cell lines, Wu

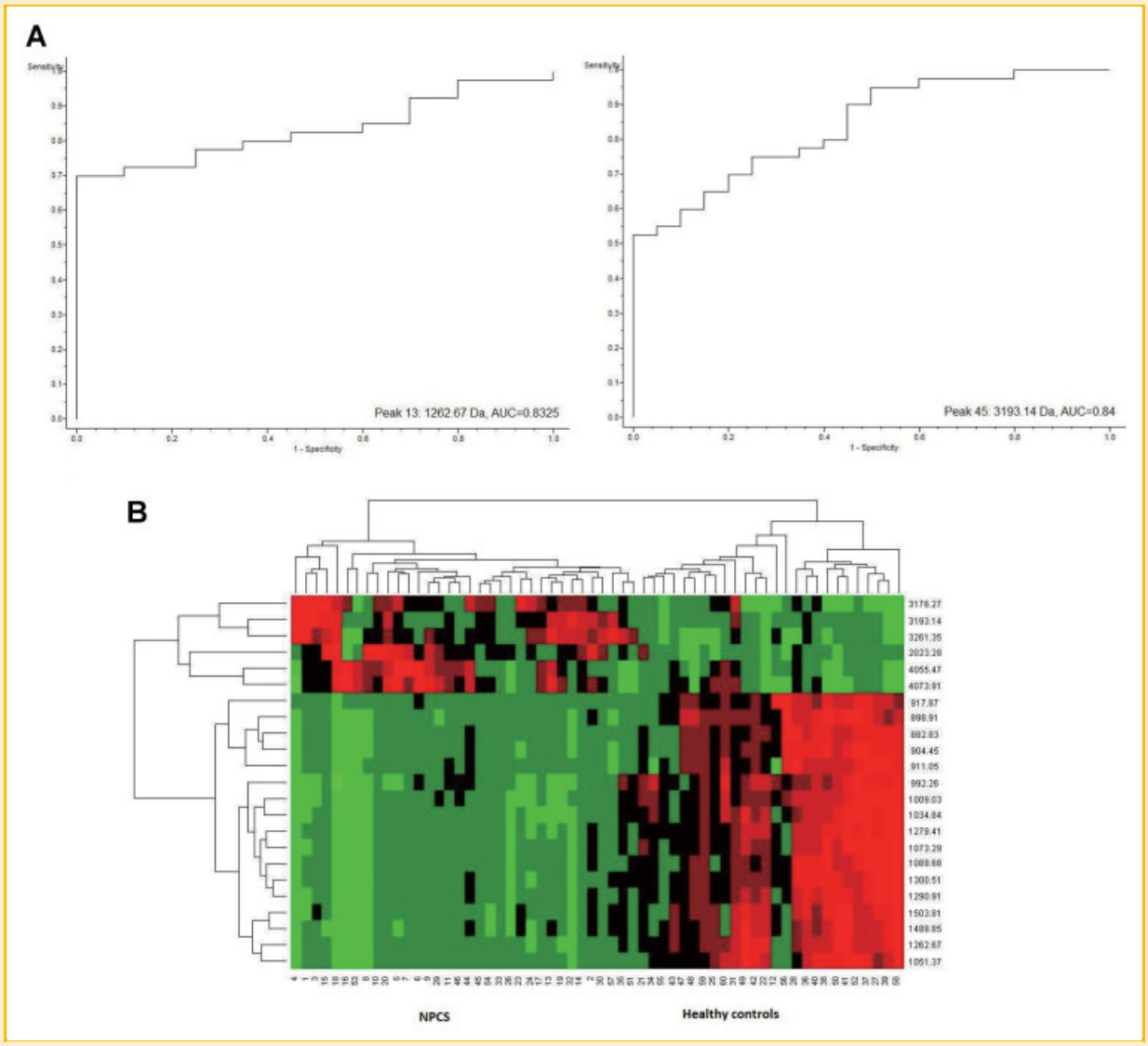


Fig. 3. Differential expression peptides performance in analysis of ROC curve and clustering methods. A. feature performance of m/z 1262.67 and m/z 3193.14 in ROC curve. The AUC of m/z 1262.67 and m/z 3193.14 was 0.83 and 0.84, respectively. B. Clustering analysis of 23 peaks with significant difference ($P < 0.001$, by *t*-test) in their distribution between NPC and control samples. The arrangement the intensities of the 23 peaks in 60 samples in binary format was by unsupervised, average-linkage hierarchical clustering using standard correlation as a distance metrics between the NPC group and the control group. Columns represent samples; rows are m/z peaks as indicated by the average molecular weight. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

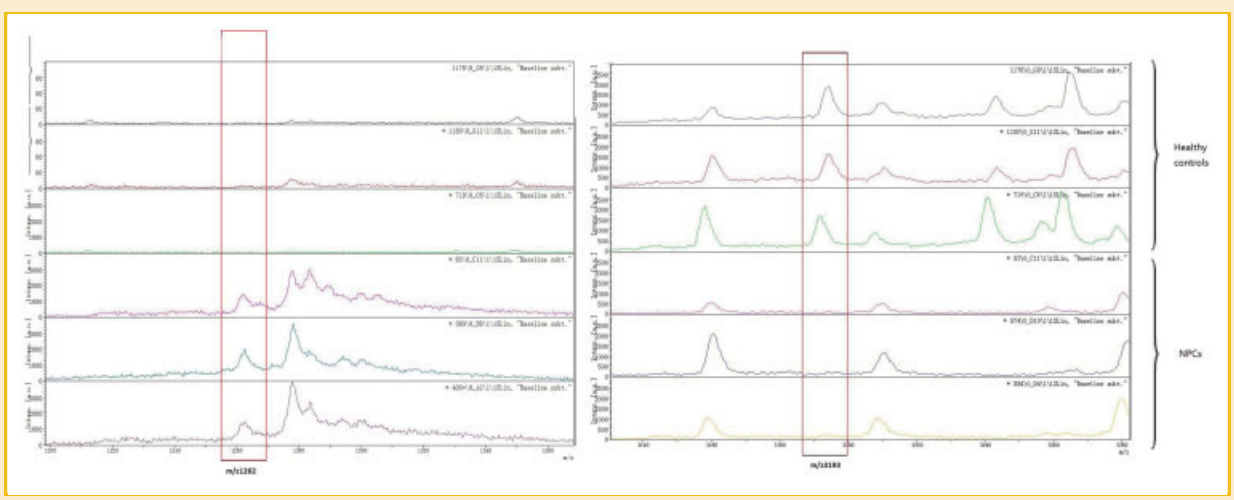


Fig. 4. Representative spectra of two peptides randomly obtained from three samples of two groups, cancer and healthy control group. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

TABLE IV. Performance of the GA Model of NPC in the Training Set and Independent Validation Sets

| | Training set | Single center validation set | Multicenter validation set |
|---|---|---|---|
| Sensitivity | 100% | 90.5% (19/21) | 91.9% (91/99) |
| Specificity | 100% | 88.9% (8/9) | 83.3% (20/24) |
| PPV | 100% | 95% (19/20) | 95.9% (91/95) |
| NPV | 100% | 80% (8/10) | 71.4% (20/28) |
| Accuracy | 100% | 90% (27/30) | 90.2% (111/123) |

et al. identified three biomarker for NPC diagnosis [fibronectin, Mac-2-binding protein (Mac-2 BP), and plasminogen activator inhibitor 1 (PAI-1)] [Wu et al., 2005]. Up-regulated cytokeratin 19 (CK19) and Erb3-binding protein (EBP1) were observed in NPC versus normal tissue, they may be utilized for NPC diagnosis [Xiao et al., 2007]. (ii) Monitors for treatment response. Doustjalali et al.'s [2006] study suggested ceruloplasmin could be potential marker for treatment response monitoring of NPC, because it was highly expressed in the NPC sera, but was back to normal after treatment for 6 months. Using proteinchip profiling analysis and immunoassay, increasing serum amyloid A (SAA) protein correlated with recurrence and decreasing correlated with response to chemotherapy, and it could be a useful biomarker to monitor relapse of NPC [Cho et al., 2004]. On the basis of results that the down-regulation of 14-3-3σand maspin and the up-regulation of GRP78 and Mn-SOD were significantly different between NPC cell line CNE-2 and CNE2-IR (a radioresistant subclone cell line) using proteomic methods and immunoassay, they could be potential biomarkers for predicting NPC response to radiotherapy [Feng et al., 2010]. (iii) Biomarkers for prognosis. Mo et al. [2005] reported that expression of Tiam1 in NPC tissue was higher than in normal tissue. This data suggested that the overexpression of the Tiam1 correlated invasion and metastasis of NPC. Tai et al. found Rsf-1 overexpression was associated with adverse prognosticators and therapeutic response in NPC, and represents a potential prognostic biomarker [Tai et al., 2012]. In our study, we used MALDI TOF MS method to detect and compare the level of FGA peptides between the NPC group and the control group, and we found levels of FGA fragments were significantly different between the two groups. The accuracy of the NPC diagnostic model with 4 FGA peptides was all above 90% which was respectively

TABLE V. Identification of Sequences of Peptide Differentially Expressed Between NPC Group and Controls

| m/z | Peptide name | Peptide sequences |
|---|---|---|
| 882.83 | Unknown peptide-identification failure | |
| 898.91 | Unknown peptide-identification failure | |
| 904.45 | FGA Isoform 1 of Fibrinogen alpha chain precursor | D.FLAEGGGVR.G |
| 911.05 | Unknown peptide-identification failure | |
| 917.87 | Unknown peptide-identification failure | |
| 992.26 | Unknown peptide-identification failure | |
| 1009.03 | Unknown peptide-identification failure | |
| 1034.84 | Unknown peptide-identification failure | |
| **1051.37** | FGA Isoform 1 of Fibrinogen alpha chain precursor | G.DSTFESKSY.K |
| 1073.29 | FGA Isoform 1 of Fibrinogen alpha chain precursor | E.GDFLAEGGGVR.G |
| 1089.68 | Unknown peptide-identification failure | |
| 1117.83 | INTU Isoform 2 of PDZ domain-containing protein 6 | Y.PMSEASQKLK.S |
| **1262.67** | FGA Isoform 1 of Fibrinogen alpha chain precursor | S.GEGDFLAEGGGVR.G |
| 1279.41 | TUBB1 Tubulin beta-1 chain | R.LHFFMPGFAPL.T |
| 1290.91 | F2 Prothrombin precursor (Fragment) | T.SEYQTFFNPR.T |
| 1300.51 | Unknown peptide-identification failure | |
| 1330.17 | MBP Isoform 1 of Myelin basic protein | R.TQDENPVVHFF.K |
| 1489.85 | FGA Isoform 1 of Fibrinogen alpha chain precursor | K.TFPGFFSPM*LGEF.V |
| 1503.81 | AHSG Alpha-2-HS-glycoprotein precursor | G.VVSLGSPSGEVSHPR.K |
| 1531.34 | FGA Isoform 1 of Fibrinogen alpha chain precursor | T.ADSGEGDFLAEGGGVR.G |
| 1866.37 | LOC653879 similar to complement component 3 | R.SSKITHRIHWESASLL.R |
| 1885.44 | FGA Isoform 1 of Fibrinogen alpha chain precursor | R.HRHPDEAAFFDTASTGK.T |
| 1897.67 | ALB Uncharacterized protein ALB | R.RHPYFYAPELLFFAK.R |
| 1946.58 | EZR Ezrin | K.IAQDLEMYGINYFEIK.N |
| 2023.28 | LOC653879 similar to complement component 3 | R.SSKITHRIHWESASLLR.S |
| 2082.76 | KNG1 Isoform HMW of Kininogen-1 precursor | K.HNLGHGHKHERDQGHGHQ.R |
| 2660.78 | FGA Isoform 1 of Fibrinogen alpha chain precursor | A.DEAGSEADHEGTHSTKRGHAKSRPV.R |
| 2883.62 | FGB Fibrinogen beta chain precursor | R.GHRPLDKKREEAPSLRPAPPPISGGGY.R |
| 3144.86 | ITIH4 71 kDa protein | M.NFRPGVLSSRQLGLPGPPDVPDHAAYHPF.R |
| 3176.27 | CLTC Isoform 1 of Clathrin heavy chain 1 | K.WISLNTVALVTDNAVYHWSMEGESQPVK.M |
| **3193.14** | FGA Isoform 1 of Fibrinogen alpha chain precursor | K.SSSYSKQFTSSTSYNRGDSTFESKSYKM.A |
| 3208.98 | FGA Isoform 1 of Fibrinogen alpha chain precursor | K.SSSYSKQFTSSTSYNRGDSTFESKSYKM*.A |
| 3228.62 | Unknown peptide-identification failure | |
| 3241.84 | FGA Isoform 1 of Fibrinogen alpha chain precursor | K.SYKMADEAGSEADHEGTHSTKRGHAKSRPV.R |
| 3261.35 | FGA Isoform 1 of Fibrinogen alpha chain precursor | K.SSSYSKQFTSSTSYNRGDSTFESKSYKMA.D |
| **4055.47** | FGA Isoform 1 of Fibrinogen alpha chain precursor | T.SYNRGDSTFESKSYKMADEAGSEADHEGTHSTKRGHA.K |
| 4073.91 | Unknown peptide-identification failure | |
| 4092.2 | FGA Isoform 1 of Fibrinogen alpha chain precursor | R.GDSTFESKSYKMADEAGSEADHEGTHSTKRGHAKSRPV.R |
| 4111.08 | FGA Isoform 1 of Fibrinogen alpha chain precursor | R.GDSTFESKSYKM*ADEAGSEADHEGTHSTKRGHAKSRPV.R |
| 4211.24 | GSPT2 Eukaryotic peptide chain release factor GTP-binding subunit ERF3B | K.EQSDFCPWYTGLPFIPYLDNLPNFNRSIDGPIRLPI.V |
| 4230.02 | Unknown peptide-identification failure | |
| 4301.95 | Unknown peptide-identification failure | |
| 5340.01 | FGA Isoform 1 of Fibrinogen alpha chain precursor | K.SSSYSKQFTSSTSYNRGDSTFESKSYKMADEAGSEADHEGTHSTKRGHA.K |

m/z value is isotopic molecular weight; the four peptides of NPC model were in bold.

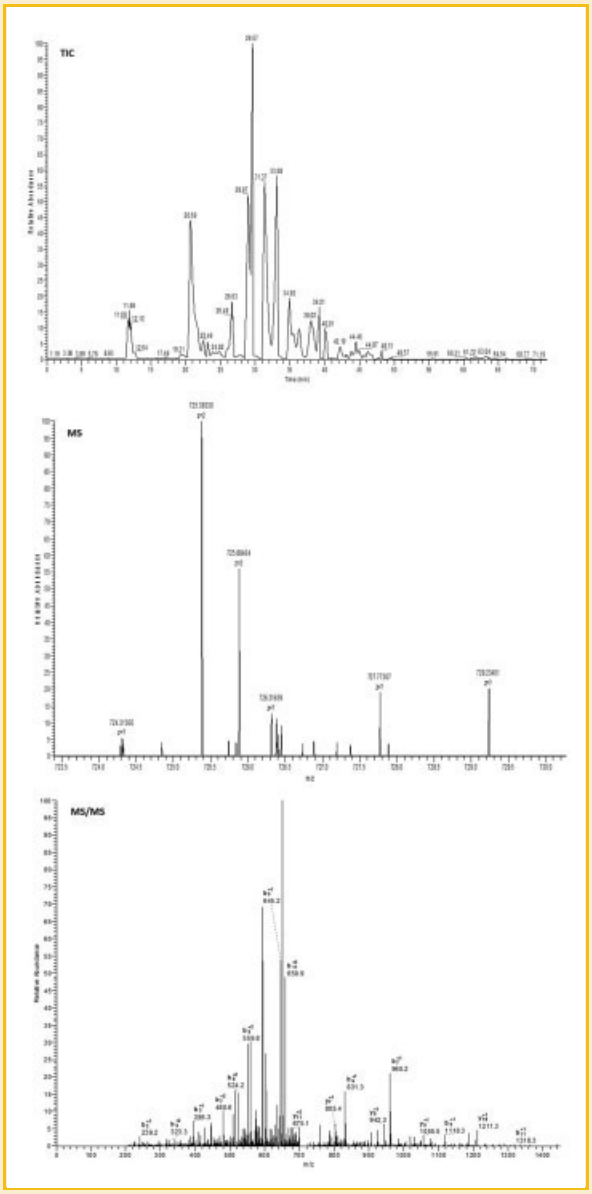


Fig. 5. A representative LTQ-Orbitrap-MS sequencing result of m/z 1262.67.

validated in the single center set of Sun Yet-sen cancer center and in the multicenter set of seven hospitals.

As we know, FGA is a protein that in human is encoded by the FGA gene, and is the component of fibrinogen, a blood-borne glycoprotein composed of three pairs of non-identical polypeptide chains. Why is the FGA related to NPC ? We think the mechanism underlying the close correlation between FGA and NPC as follow: NPC is one of the malignant tumors. Currently, there have been many considerable studies on relationship between high level of Fg and malignancy, such as pancreas carcinoma, renal cell carcinoma, endometrial carcinoma, osteosarcoma, esophageal squamous cell carcinoma, etc. Preston et al. found high level of fibrinogen in circulating concentration in pancreas carcinoma was the result of increased synthesis of fibrinogen, a acute phase plasma protein, associated with an ongoing inflammatory response to tumor

TABLE VI. Performance of the GA Model of NPC in the NPC Group and Other Cancer Group

| | NPC | Other cancers |
|---|---|---|
| False positive rate | 4/24 (16.7%) | 16/41 (39%) |
| False negative rate | 8/99 (8.1%) | 25/41 (61%) |

[Preston et al., 1998]. Lord's, Seebacher et al.'s, and Voland et al.'s studies suggested that tumor cells could promote coagulation process by interacting with endothelial cells and platelets, then by releasing active biological substances that activate platelets, whish lead to high level of Fg in the cancer blood. In our study, levels of FGA in NPC patients was different from healthy controls, this result could be also caused by the mentioned-above reasons of inflammatory response to NPC and hypercoagulable state in NPC patients. Secondly, we used GA to establish the discriminant model of NPC diagnosis. The accuracies of the GA model were above 90% in the NPC group, and the FPR and FNR were higher in the other cancer set (Tables IV and VI). These results indicated that the model in our manuscript was more suitable for the diagnosis of NPC than other cancers and had high sensitivity and specificity. It was the possible reasons that different tumor cells could induce the secretion of different proteases which lead to different proteolytic degradative patterns in the serum peptidome, the latter provided important information for detection and classification of specific cancer. Villanueva's studies showed differential protease activities confer bladder cancer-specific, prostate cancer-specific, and thyroid cancer-specific serum peptidome patterns [Villanueva et al., 2006a, 2006b], in our study, the protease induced by NPC cells confer the tumor type-specific serum pattern of FGA peptides (shown in Fig. 6). Thirdly, from the perspective of the clinical biological behavior of malignant tumor, Palumbo et al.'s studies

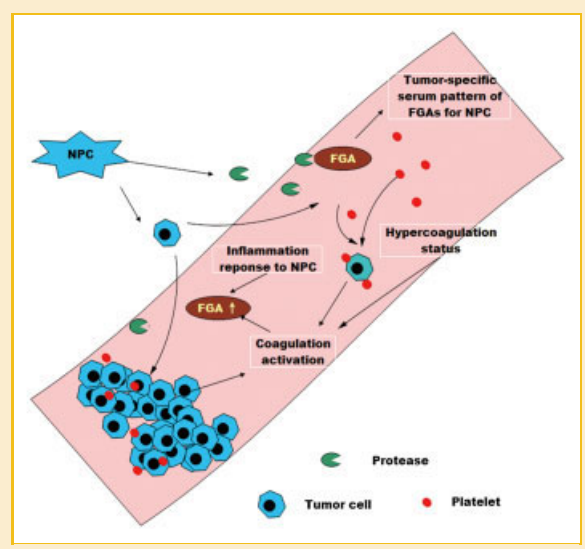


Fig. 6. Possible mechanism of high FGA level in blood of NPC patients. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

found that fibrinogen, as a bridge between tumor cell and platelet, and endothelial cells, can enhance tumor cell adhesion to improve the ability of tumor cell metastasis in vitro/vivo [Palumbo et al., 2000, 2002; Takeuchi et al., 2007; Verheul et al., 2010]. The researches of Takeuchi et al., Verheul et al., and Seebacher et al. suggested that Fg was also an independent prognostic factor for the disease-free, distant-free, and overall survival in endometrial cancer, esophageal carcinoma, and renal cell carcinoma [Palumbo et al., 2000, 2002; Takeuchi et al., 2007; Verheul et al., 2010]. Whether FGA can promote NPC cell metastasis to be an independent prognostic factor of survival rate in NPC patients, it need further study to explore and confirm. [Li et al., 2006]. In addition, mutations in this gene of FGA lead to several disorders, including dysfibrinogenemia, hypofibrinogenemia, afibrinogenemia, and renal amyloidosis. The total of 160 with NPC patients and 53 healthy volunteers have not any disease with disorder of FGA mentioned before. We described the characteristic of the patient and control cohort in our study. (Table I). For decreasing the bias by smoking status, we analyzed the two group with ($n = 28$) or without ($n = 41$) smoking in NPC patients. The results indicated that there were no significant differences using Wilconxon test ($P > 0.05$). Moreover, some modification of peptides would change the peptides profiles, such like acetylated lysines and so on, but the results did not change in our study because we only obtained and utilized the molecular weight of peptide using MALDI TOF MS, not including any modification of peptides.

As a conclusion, we think that FGA fragments may serve as tumor-associated biomarkers for NPC and the diagnostic model with four peptides of 3193.14 m/z, 1051.37 m/z, 4055.47 m/z, and 1262.67 m/z was suitable to NPC. We will further perform proteomics research to find out the diagnostic difference peptide fragments used in judging recurrence and metastasis of NPC.

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