[Frontiers in Bioscience E2, 961-971, June 1, 2010]

# Identification of novel serum biomarkers for gastric cancer by magnetic bead

**Wentao Liu[1], Xiang Gao[1], Qu Cai[1], Jianfang Li[1], Zhenglun Zhu[1], Chen Li[1], Xuexin Yao[1], Qiumeng Yang[1], Ming Xiang[1], Min Yan[1], Zhenggang Zhu[1]**

[1]*Department of Surgery, Ruijin Hospital, Shanghai Jiao Tong University, Shanghai Institute of Digestive Surgery, Shanghai 200025, PR China*

## TABLE OF CONTENTS



## 1. ABSTRACT

Early diagnosis and early treatment is known to improve prognosis for gastric cancer. Magnetic affinity beads can be used to extract peptides from un-fractionated serum samples. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) can detect the presence and the molecular mass of peptides. MALDI-TOF-MS mass spectra of peptides and proteins were generated after WCX CLINPROT bead fractionation of 62 gastric cancer serum samples. The discovery set consisted of 44 samples while the validation set was 18 serum samples. The spectra were analyzed statistically using flexAnalysisTM and Clin-ProtTM bioinformatic software. The six most significant peaks were selected out by ClinProTool software and utilized to train a Supervised Neural Network to identify gastric cancer sera from control sera. The sensitivity and specificity of the model when tested on the validation set were 100% and 75%, respectively. A set of 6 peptides that can be used to distinguish serum from gastric cancer patients with good sensitivity and specificity were identified, and these peptides may be useful biomarkers to distinguish cancer individuals who may benefit from radiologic or endoscopic examination.

## 2. INTRODUCTION

In recent years, proteomics has become a broadly developing technique in the field of biotechnology. A primary goal of proteomics is the discovery of biomarkers for various human disease conditions, especially cancers. Plasma and serum are considered to be the source of choice in molecular diagnostics (1). However, although readily accessible from patients, plasma proteome analysis is influenced by a lot of major components present, such as serum albumin, immunoglobulin, etc., the predominance of which raises detection problems for the less abundant marker species (2). Several researchers have tried to provide early cancer detection via sophisticated software processing of serum/plasma mass spectrometry profiles, where the result is typically based on a pre-trained artificial intelligence system decision (3-5). Serum biomarkers for gastric cancers have been identified by two-dimensional gel electrophoresis (2D PAGE) (6), antibody microarray (7), and surface enhanced laser desorption ionization mass spectrometry (SELDI-MS) (8, 9). However, among a huge amount of biomarkers discovered by other technology, only a few of them have been identified due to the technique limitation in supporting direct identification on chip using a ProteinChip Array. In fact, identification of these

candidates will not only assist in exploring the mechanism of carcinogenesis, but also facilitate the development of more traditional multiprotein antibody arrays for the early detection of cancer.

Gastric cancer is the fourth most common cancer in the world. Almost two-thirds of cases occur in less developed countries with high incidences in East Asia, Eastern Europe, and Central and South America (10). Early diagnosis and early treatment is known to improve prognosis, but a lack of specific biomarkers (11) means that diagnosis by upper gastrointestinal radiography, endoscopy and biopsy is often too late. Consequently, these tumors tend to be advanced and result in a low (< 10%) five-year survival rate.

Currently, the screening of cancer biomarkers is a hot field in serous proteomics because serous proteins often serve as indicators of diseases and are therefore rich sources for biomarker discovery. Due to the reduced costs associated with affinity bead–based purification development, these proteomic procedures have become suitable for general MS analysis. This method uses different chemical chromatographic surfaces on an outer layer of magnetic beads to selectively purify certain subsets of proteins, thus allowing unbound impurities to be removed by washing with buffers. Proteins bound to the magnetic beads are then eluted, diluted, and directly analyzed by MALDI-TOF-MS. Bioinformatics algorithms are used to align and integrate hundreds of mass data points from large numbers of samples. The technical performance of affinity bead purification is similar to that of ELISA, and it can be used to process many samples in parallel. This approach is sensitive and fast, which are features essential for clinical use. Moreover, the cost is low, and further protein identification can be easily performed from the eluted material without the need for complex purification. (12-14). A clinically-useful circulating tumor marker with good sensitivity and specificity for diagnosis and monitoring of gastric cancer progression may help to decrease the current morbidity rates for patients diagnosed with gastric cancer. We describe potential biomarkers identified through the use of affinity bead purification and MALDI-TOF-MS.

## 3. MATERIAL AND METHODS

### 3.1. Patients and blood sample preparation

Serum samples were collected from control patients and patients with potentially-resectable gastric cancer. Blood was collected preoperatively in glass tubes without additive (BD Vacutainer™ Franklin Lakes, NJ) and was allowed to clot at room temperature for 40 min. Serum was separated by centrifugation at 2000 rpm for 15 min, immediately split into 200µl aliquots and frozen at −80 °C until analysis. The time from collection to frozen storage was no more than 60 min. All patient data were anonymized. The processing, collection and storage protocols for all individuals were identical.

The discovery set used to train the neural network comprised of 44 serum samples: 28 from patients with histologically-confirmed gastric cancer (17 male, 11 female, median age 65 years), and 26 from control individuals (16 male, 10 female, median age 60). The Verification set was not used in training and comprised of eighteen-samples: 10 from patients with histologically confirmed gastric cancer (7 male, 3 female) and eight from control individuals (5 male, 3 female). Researchers and operators were blinded with regard to the verification set diagnoses. All control subjects were free of malignant disease and denied a personal or family history of cancer. In all gastric cancer patients, diagnosis was histologically-confirmed and staging of the tumors was performed according to routine cancer patient management protocols. This study was performed according to the guidelines of the Ethics Committee of Shanghai Jiao Tong University School of Medicine.

### 3.2. Serum protein fractionation

All samples were assayed in duplicate. Serum samples were thawed and purified using chemically coated magnetic WCX (Weak cation exchange) beads (Bruker Daltonics,). 10 µl of Serum was mixed with 5 µl of beads and samples were purified through 3 steps (binding, washing, and elution) in accordance with the manufacturer's protocol. Each incubation step took 1 min. Elution was carried out with 5 µl of elution buffer and the purified material was 8-fold diluted with the elution solution prior to MS analysis.

### 3.3. Mass spectrometry analysis to profiling serum proteome

For MALDI-TOF-MS analysis, 1 µL of the above-diluted purified serum was mixed with 0.5 µl of matrix solution (2 g/L α-cyano-4-hydroxycinnamic acid, and 1% formic acid in 50% acetonitrile) and allowed to dry onto the MALDI sample plate (600 µm AnchorChip™, Bruker Daltonics Company). Two peptides were also included in the matrix solution for internal calibration: 10 pmol/mL angiotensin II and 10 pmol/mL ACTH18-39 (Bruker Daltonics). Laser desorption was targeted randomly on the sample plate and samples were measured using an Autoflex II MALDI-TOF mass spectrometer (Bruker Daltonics) operated in positive ion linear (reflection) mode. Ionization was achieved by irradiation with a 50 Hz nitrogen laser. Spectra were the mean of 100 ionizations with fixed laser power in linear geometry mode and mass maps were obtained in reflectron mode. The spectra were calibrated externally with a mixture of protein/peptide standards in the range of 1000 to 12 000 Da (Bruker Daltonics). For data bank analysis, all spectra were processed by automatic baseline subtraction, peak detection, recalibration, and peak area calculations were performed according to the predefined parameter settings. The criteria for peak detection were: Signal/Noise (S/N) ratio > 5, 2 Da peak width filter, and maximum peak number of 200. The intensities of the peaks of interest were normalized against the peak intensity of the ACTH internal standard. A ±2 Da mass accuracy for each spectrum was observed, which may be due to the geometry of varied sample positions on the AnchorChip. These mass shifts were corrected by the flexAnalysis™ software after alignment with the 2 internal standards.

**Table 1.** Mass spectral characteristics of proteins/peptides with differential expression between gastric cancer and control subjects (p<0.005)

| m/z | D Ave | p Value | Ave N | Ave T | SD N | SD T |
|---|---|---|---|---|---|---|
| 5248.49 | 33.37 | < 0.000001 | 33.78 | 67.15 | 6.99 | 23.48 |
| 5754.25 | 15.76 | < 0.000001 | 11.46 | 27.23 | 3.12 | 11.55 |
| 4268.05 | 55.31 | 1.31E-06 | 89.25 | 33.94 | 27.97 | 17.13 |
| 5809.75 | 49 | 7.72E-06 | 26.99 | 75.99 | 7.7 | 38.61 |
| 5480.05 | 13.47 | 1.39E-05 | 17.27 | 30.75 | 5.28 | 10.5 |
| 4118.84 | 64.62 | 0.000017 | 120.35 | 55.74 | 39.06 | 20.03 |
| 2105.18 | 18.52 | 2.55E-05 | 35.45 | 16.93 | 11.51 | 5.91 |
| 5904.83 | 1057.79 | 2.67E-05 | 499.66 | 1557.45 | 186.04 | 767 |
| 4152.83 | 20.47 | 4.76E-05 | 38.29 | 17.82 | 13.95 | 8.5 |
| 3315.62 | 32.85 | 0.000113 | 77.82 | 44.97 | 26.17 | 13.68 |
| 1450.13 | 9.3 | 0.000174 | 19.96 | 10.65 | 6.95 | 3.99 |
| 4074.25 | 16.27 | 0.000174 | 36.03 | 19.77 | 12.91 | 7.3 |
| 3507.34 | 22.28 | 0.000329 | 45.33 | 23.05 | 34.38 | 25.5 |
| 5636.53 | 10.24 | 0.000376 | 14.97 | 25.22 | 2.99 | 10.44 |
| 4091.23 | 106.32 | 0.000376 | 243.74 | 137.42 | 81.76 | 58.15 |
| 4280.65 | 60.23 | 0.000527 | 101.35 | 41.12 | 87.58 | 37.44 |
| 2545.9 | 16.43 | 0.000565 | 66.48 | 50.06 | 12.11 | 10.72 |
| 1778.75 | 17.42 | 0.00162 | 36.34 | 18.93 | 21.46 | 8.47 |
| 6049.46 | 13.54 | 0.00173 | 18.63 | 32.17 | 6.9 | 14.72 |
| 2951.97 | 42.92 | 0.00232 | 66.53 | 109.45 | 17.28 | 45.51 |
| 5963.11 | 43.96 | 0.00276 | 43.56 | 87.52 | 18.13 | 48.12 |
| 4210.16 | 265.8 | 0.00327 | 597.63 | 331.83 | 240.75 | 190.67 |
| 4964.28 | 68.89 | 0.0043 | 140.58 | 71.69 | 81.73 | 89.24 |

M/Z = The mass/charge characteristic of each protein/peptides; D Ave: difference between the maximal and minimal average peak area; p Value: p-value of Wilcoxon test (2 class) or Kruskal-Wallis (>2class); Ave N or T: peak area (intensity.) average of class N or T; SD N or T: standard deviation of the peak area average of class N or T.

### 3.4. Statistical methods, evaluation of diagnostic efficacy

All MALDI-TOF-MS spectra were analyzed with flexAnalysis™ to detect the peak intensities of interest and CLINPROT™ software to compile the peaks across the spectra obtained from all samples (Bruker Daltonics Company). This analysis allowed for discrimination between cancer and control samples. To evaluate the precision of the assay, we determined within- and between-run variations via multiple analyses of bead fractionation and MS for 2 plasma samples. For within- and between-run variation, we examined 3 peaks with various intensities. We determined within-run imprecision by evaluating the CVs for each sample, using 12 assays within a run. To assess diagnostic efficacy, we calculated the means (SD) of the peaks of interest in the control group. The cut-off value was defined as mean plus 2SD of the control samples. The sensitivity (ratio of the cancer samples with a mass intensity greater than the cutoff value to all samples in the cancer group) and specificity (ratio of control samples with a mass intensity less than the cutoff value to all samples in the control group) were analyzed accordingly. Receiver operator characteristic (ROC) curves for differentiating gastric cancer patients from control subjects were constructed by calculating the sensitivities and specificities of the biomarkers at different cut-off points. Area under ROC curves (AUC) values of 0.50.7 suggest low diagnostic accuracy, values of 0.70.9 suggest limited clinical utility and values >0.9 suggest high global diagnostic accuracy (10).

### 3.5. Identification of protein markers

Selected peptides were further purified by use of C18 beads from Nano Aquity UPLC (Waters Corporation, Milford, USA) and serially eluted with 5% and 95% acetonitrile. These peptides were identified directly by LTQ Orbitrap XL (Michrom Bioresources, Auburn, USA) analysis to obtain the peptide sequences. Peptide mass fingerprinting was performed with the International Protein Index (IPI Human v3.45 Fasta with 71983 entries) and a search of the National Center for Biotechnology Information (NCBI) protein-protein BLAST database (http://www.ncbi.nlm.nih.gov/BLAST/).

## 4. RESULTS

### 4.1. Identification of serum proteomic features associated with gastric cancer

To determine proteomic patterns and search for a specific Gastric Cancer marker, magnetic bead purified samples exhibited spectral peaks in the 1000–10,000 Da range. Typical WCX spectra for healthy and gastric cancer serum, as well as the effect of pre-processing and normalization, are shown in Figure 1. Differences in peak positions and intensities, which were later used to statistically analyze the spectra, can be seen. Using ClinprotTools ver 2.1 (Bruker Daltonic), the centroid peak detection algorithm using an S/N threshold of 2 and peak width of 5 m/z units identified 30%-40% more peaks in mouse serum spectra than human serum spectra when using comparable clotting, WCX and MALDI-MS protocols.

MALDI-TOF analysis of the normal and gastric cancer patients resulted in 94 distinguishable peaks available in the 1,000 to 10,000 m/z range, with 23 peaks having differential expression and statistical significance P<0.01 (shown in Table 1). To assess the diagnostic efficacy, the mean (SD) masses of the 23 peaks in the control group were calculated. Two significantly differently expressed proteins of 5248.49Da and 5754.25Da were identified with high expression in the gastric cancer sera as compared to controls (shown in

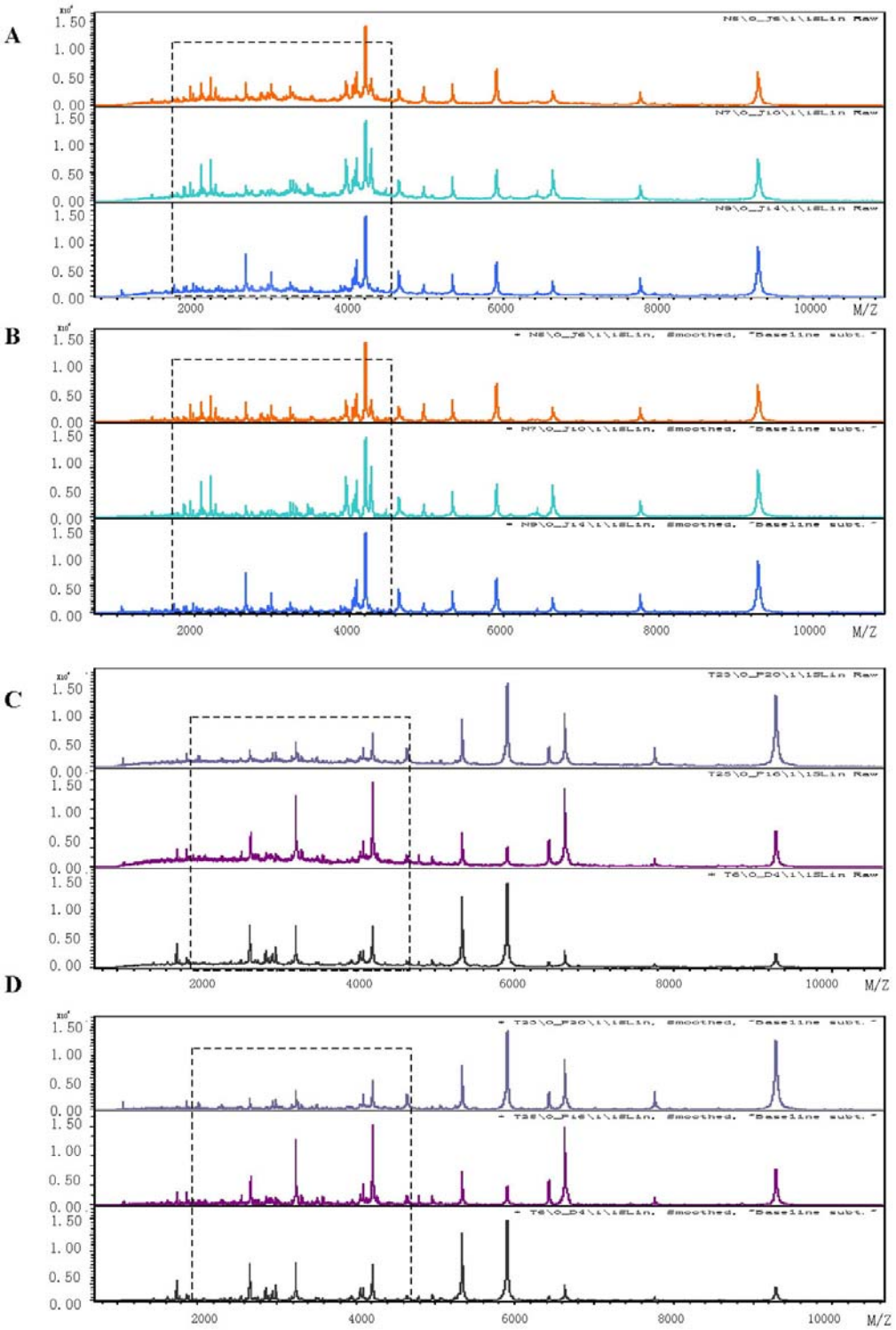


**Figure 1.** Comparative mass spectrum. Plots A and C show the untreated profile of the control (N group) and the gastric cancer group (T group). Plots B and D show the treated profile of the N group and T group after de-noising by CLINPROT software. Each plot (A to D) shows three lines which represent three different serum sample profiles. In the dotted rectangles, the base lines on B and D are observed as significantly smoother and flatter as a result of the de-noising process when compared to those in A and C.

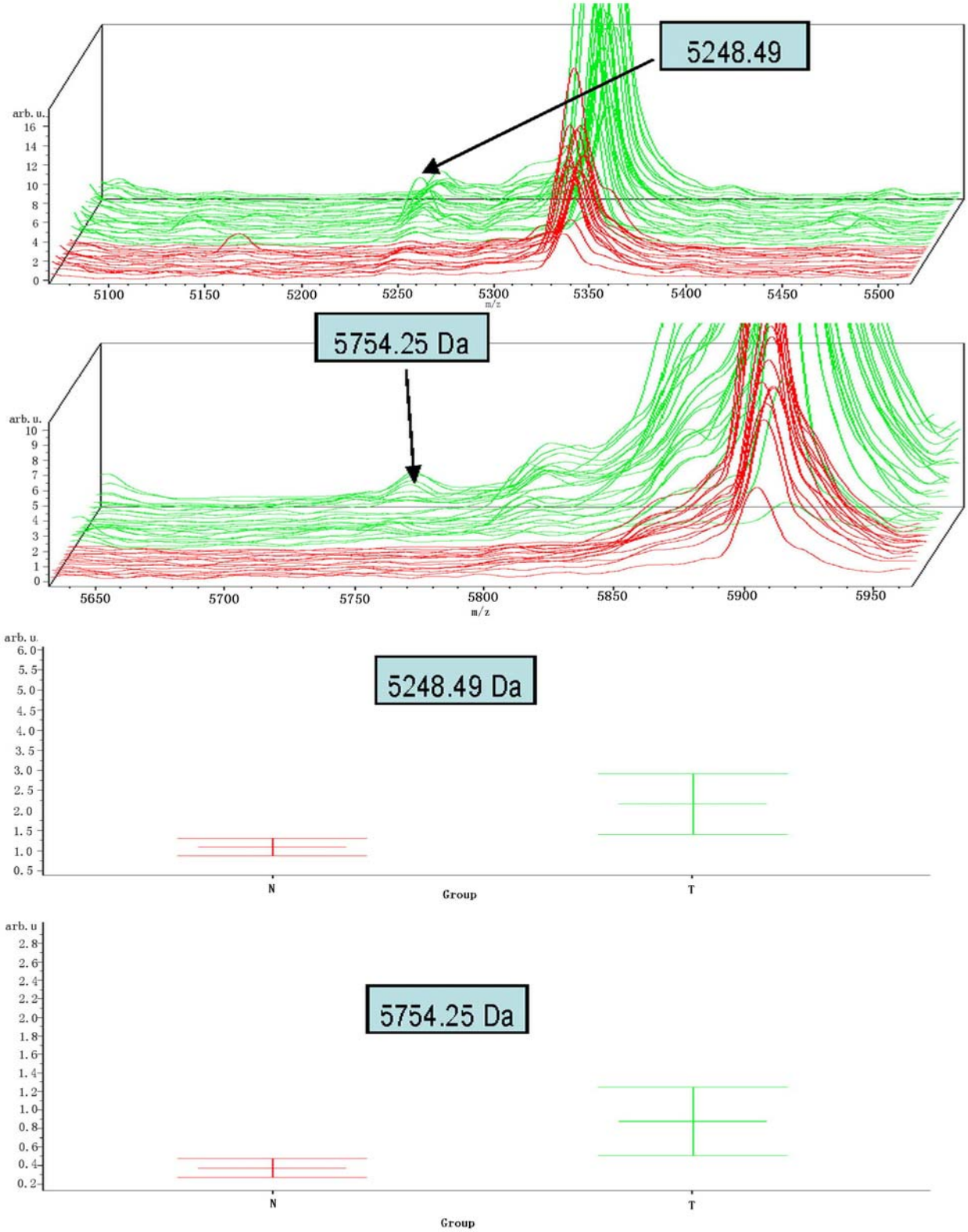


**Figure 2.** Mass spectra profiles of proteins/peptides with masses 5248.49D and 5754.25Da. In both plots, the control group (N) is shown in red and the gastric cancer group (T) in green. In both cases, the mean ± SD peptide concentration (shown in lower box-plot) is higher in the tumor group than in the control group.

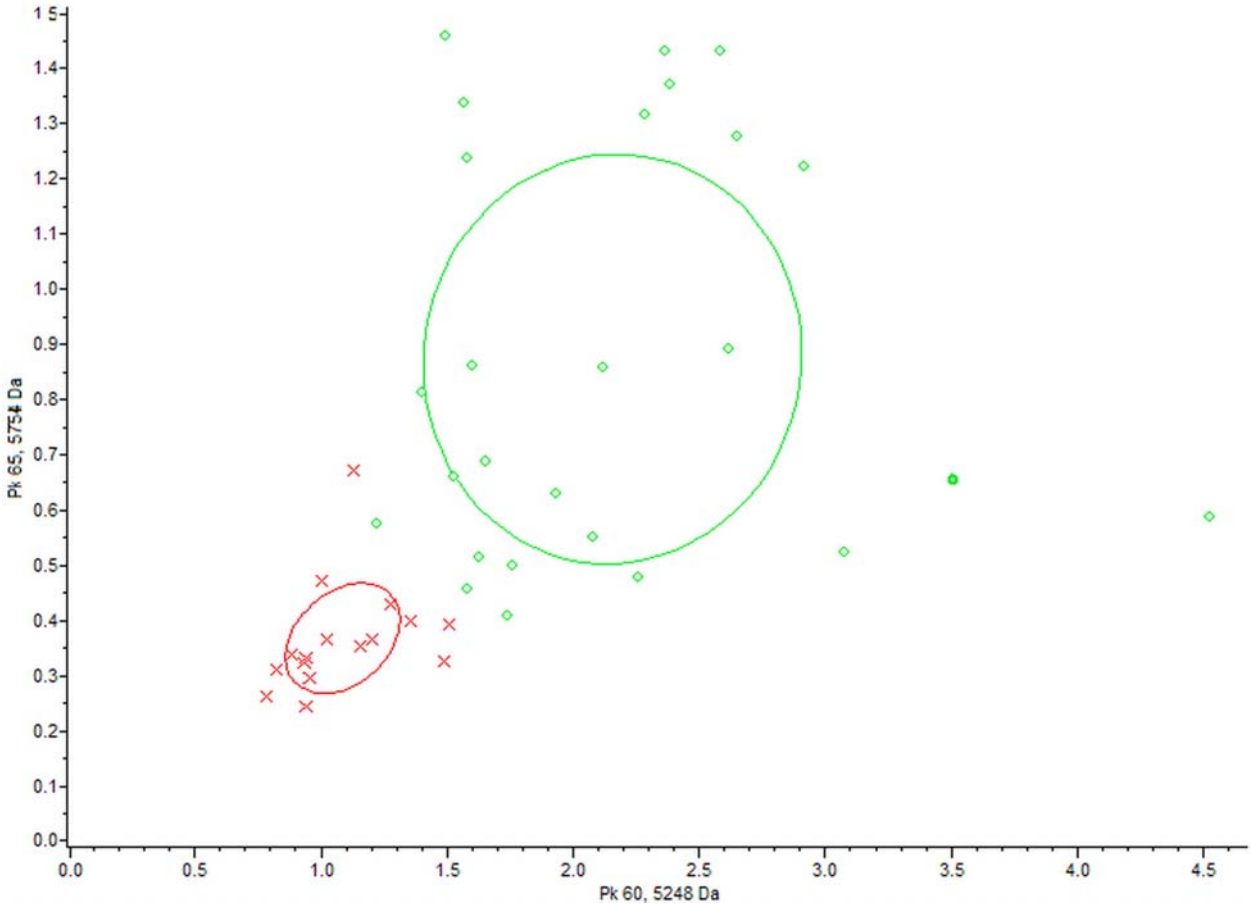


**Figure 3.** Bivariate plot of two protein peaks 5248.49Da and 5754.25Da showing significant distinction between distribution in control and tumor data sets. Each of the green dots and the red crosses showed the sample of T group and N group for discovery set respectively.

Figure 2). These two peaks appeared to have significant discriminatory potential (shown in Figure 3).

### 4.2. Sensitivities and specificities of the biomarkers by receiver operating characteristic (ROC) curve analysis

A receiver operating characteristic (ROC), or simply ROC curve, is a graphical plot of the sensitivity vs. (1-specificity) for a binary classifier system as its discrimination threshold is varied. The ROC can also be represented equivalently by plotting the fraction of true positives (TPR = true positive rate) vs. the fraction of false positives (FPR = false positive rate). Also known as a Relative Operating Characteristic curve, because it is a comparison of two operating characteristics (TPR & FPR) as the criterion changes. ROC curves were constructed for each of the 23 proteomic features by calculating the sensitivities, specificities and accuracies of the biomarkers at different cut-off points for differentiating gastric cancer patients from control subjects. The areas under the ROC curves of these proteomic features were between 0.77 and 0.98 in the discovery set. Table 2 shows the specificity and sensitivity of the different proteins. The proteins with peaks 4268.05, 5248.49 and 5904.83 showed the highest AUCs (> 0.95) (shown in Figure 4).

### 4.3.Establishment and validation of gastric cancer predicting model

A Supervised Neural Network (SNN) in CLINPROT was trained with the detected peaks from the discovery set to generate cross-validated classification models. The best predicting model resulted in a recognition capability between gastric cancer and controls of 100%. Six MALDI-TOF peaks (4268.05m/z, 5636.53m/z, 5248.49m/z, 2933.15m/z, 1450.13m/z and 1349.4m/z) were used in this best classification model. The neural network calculated a cross-validity estimate, which was 90.59%. To validate the model accuracy, the validation set data was tested using the optimum model. The sensitivity and specificity of the diagnostic model was 100% and 75% respectively.

### 4.4. Identification of the cancer markers

With this bead-based proteomic technology, we found several potential gastric cancer markers. A highly sensitive (75%) and specific (100%) marker occurred at

**Table 2**. ROC analysis of different expressed proteins

| Mass | Specificity | Sensitivity | Cutoff | ROC AUC |
|---|---|---|---|---|
| 5248.49 | 1.000 | 0.846 | 47.765 | 0.983 |
| 5754.25 | 0.938 | 0.731 | 17.701 | 0.964 |
| 4268.05 | 0.750 | 1.000 | 68.186 | 0.950 |
| 5809.75 | 0.938 | 0.769 | 42.380 | 0.928 |
| 5480.05 | 0.938 | 0.500 | 27.835 | 0.918 |
| 4118.84 | 0.688 | 0.962 | 95.787 | 0.913 |
| 2105.18 | 0.750 | 0.962 | 28.748 | 0.906 |
| 5904.83 | 1.000 | 0.846 | 871.733 | 0.904 |
| 4152.83 | 0.563 | 0.962 | 34.824 | 0.894 |
| 3315.62 | 0.625 | 0.962 | 72.320 | 0.880 |
| 1450.13 | 0.500 | 0.962 | 18.632 | 0.870 |
| 4074.25 | 0.625 | 0.962 | 34.366 | 0.870 |
| 3507.34 | 0.125 | 0.962 | 74.056 | 0.858 |
| 5636.53 | 0.938 | 0.615 | 20.949 | 0.853 |
| 4091.23 | 0.563 | 1.000 | 253.712 | 0.853 |
| 4280.65 | 0.250 | 0.962 | 116.003 | 0.846 |
| 2545.9 | 0.375 | 1.000 | 71.505 | 0.844 |
| 1778.75 | 0.375 | 0.962 | 35.857 | 0.822 |
| 6049.46 | 1.000 | 0.462 | 32.424 | 0.820 |
| 2951.97 | 0.938 | 0.538 | 101.096 | 0.813 |
| 5963.11 | 0.938 | 0.577 | 79.821 | 0.808 |
| 4210.16 | 0.375 | 0.962 | 713.169 | 0.803 |
| 4964.28 | 1.000 | 0.000 | -22.875 | 0.796 |

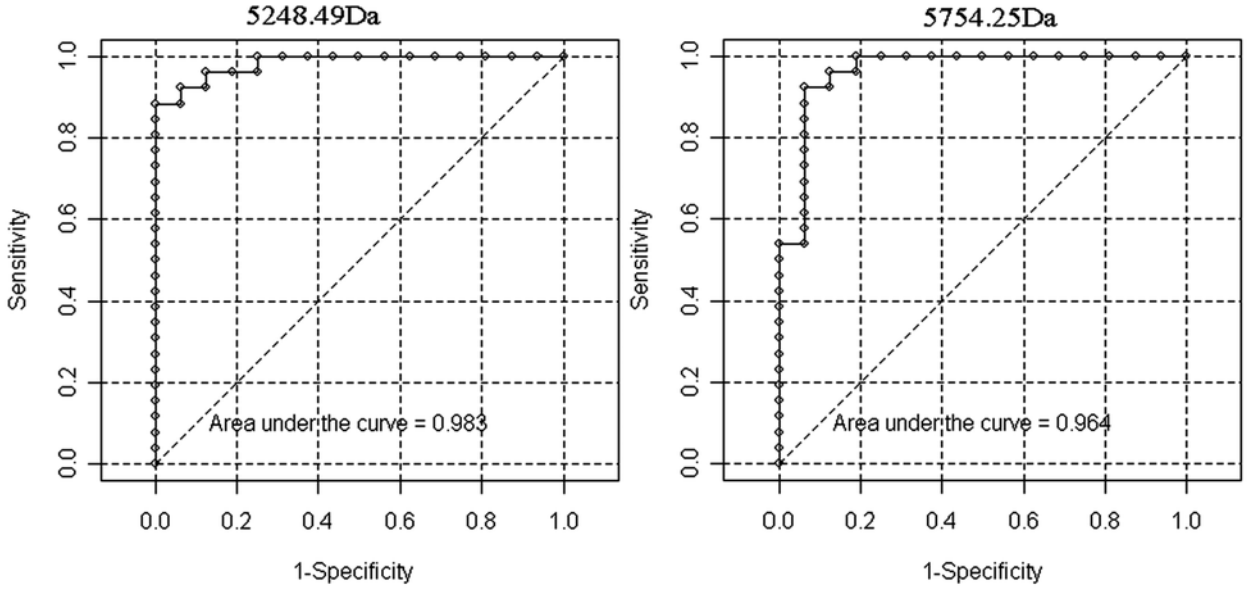


**Figure 4.** Receiver operator characteristics (ROC) curves generated with the 5248.49Da and 5754.25Da used to distinguish gastric cancer from normality. The areas under the curve (AUC) were 0.983 for the 5248.49Da protein peak and 0.964 for the 5754.25Da protein peak.

4268 Da (p=1.31E-06), which we further identified as having the highest AUCs (> 0.95). After fractionation by Nano Aquity UPLC (Waters Corporation, Milford, USA), the eluted gastric cancer plasma samples were further purified by C18 beads with diameters of 5μm and 3. 5μm, then serially eluted with 5% and 95% acetonitrile. Samples were then subjected to LTQ Orbitrap XL MS/MS (Michrom Bioresources, Auburn, USA) analysis. Marker 4268Da was significantly enriched. This acetonitrile eluate was further subjected to TOF MS/MS analysis. The MS fingerprint was subjected to International Protein Index (IPI Human v3.45 Fasta with 71983 entries) searching for peptide sequence and further analyzed using the NCBI database for protein identification. The sequence was determined to be PFTQCVTKGSFKAQWQETTYNLFTFCCLFLLPLTAM, which matches 36 of 178 amino acids in the fragment of Isoform 1 of Putative gonadotropin-releasing hormone II receptor (GNRHR2, shown in Figure 5).

## 5. DISCUSSION

We directly profiled protein/peptide patterns from affinity bead-purified plasma samples with MALDI-TOF-MS and determined several markers that differentiated gastric cancer from control samples with high sensitivity (>90%) and specificity (>90%). However, the number of specimens analyzed in our study was relatively and thus may limit the validity of

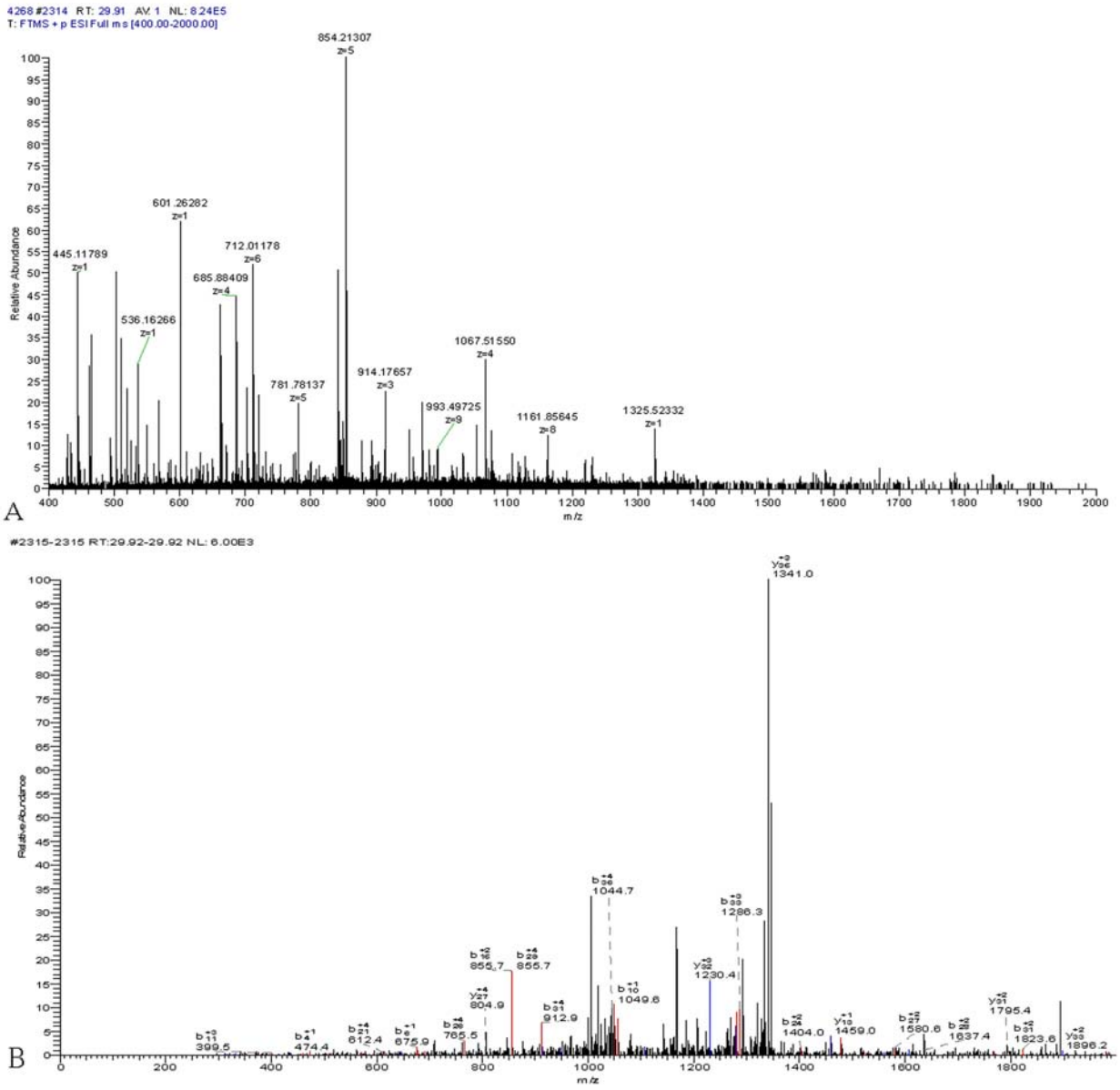


**Figure 5**. Identification of proteins by TOF/TOF analysis. A, single scan fragmentation spectrum of the (M +15H) $^{15+}$ charge state of sera acquired in the Orbitrap at 100,000 resolution (1067.51(4+), 854.21(5+), 712.01(6+)); B, MS/MS fragmentation spectrum analyzed in the Orbitrap for ion of 1067Da.

these markers and their subsequent utility in clinical diagnostics. As a result, further independent validation studies with a larger sample size are needed to determine the utility of this marker for diagnosis, which is currently being undertaken in our lab.

As the key to better prognosis for gastric cancer patients is early detection and treatment, tumors confined to the mucosa or sub mucosa identified at an early stage result in a five-year survival rate of over 90% (16). In late detection, prognosis is much worse, however, a lack of convenient diagnostic markers makes late diagnosis and consequent poor prognosis more common. Tumor antigens either in the serum (CEA, CA19.9, CA72-4 and CA50) or gastric juice (CEA, CA19.9 and fetalsulfoglycoprotein) have low sensitivity and specificity and are not clinically useful (17, 18). Meanwhile, two general categories of cellular proteins are released into the circulation: large proteins that are actively secreted and low-molecular-weight (LMW) proteins that enter the blood passively from cellular degradation or cleavage (19, 20). There is great deal of interest in the LMW region as a source of diagnostic information, particularly substances smaller than 20 kDa. Degradation by endo-protease activity is a proposed mechanism to account for the abundance of

LMW peptides found by direct MS analysis of plasma. In our results, the marker of 4268Da that was later identified by MS/MS may result from this mechanism, and further research about this is ongoing in our lab.

High-throughput serum proteomic techniques including 2D-PAGE, SELDI-MS, and HCLP have been used to study candidate serum biomarkers for early gastric cancer detection. 2D-PAGE enables simultaneous visualization of relatively large portions of the proteome and has been used to identify gastric cancer markers (6), however, 2D-PAGE may not be clinically useful, due to its complexity. Several diagnostic models for gastric cancer have been described using the SELDI technique (27-31). Poon et al (11) demonstrated a model with a specificity of 95% and a sensitivity of 83%. SELDI, however, has problems of stability and reproducibility and therefore may not be suitable for routine clinical use, and it is also not possible to identify these selected out candidate biomarkers with this technique either. CLINPROT MALDI-TOF-MS, using magnetic bead affinity purification is highly accurate and reproducible, with a sensitivity, which allows high throughput and is compatible with the identification of proteins (12, 22-24). This technique has been applied to the identification of many proteins associated with various solid organ malignancies, such as pancreatic cancer (25), nasopharyngeal cancer (14), and breast cancer (26). For gastric cancer, Ebert et al (25) used MALDI-TOF mass spectrometry after pre-fractionation of sera with magnetic hydrophobic C8 coated beads. A peptide fragment for m/z value 1465.64 Da was found to be highly elevated in cancer sera and was identified as fibrinopeptide A. We found 94 distinguishable peaks in the 1,000 to 10,000 m/z range, with 23 peaks having statistically significant differential expression ($P<0.005$). The normalized peak intensity of each feature was analyzed by ROC curve. Using the SNN analysis package in the CLINPROT software, six particularly significant peaks were selected from the discovery set data and were used to generate a diagnostic model which was applied to data from the validation set. Sensitivity and specificity for the diagnosis of gastric cancer estimated on this set was 100% and 75% respectively. We further identified three peptide fragments with respective m/z values of 4268.05, 5248.49 and 5904.83 with high accuracy (AUC > 0.95) by ROC analysis. These protein/peptide fragments with high specificity and sensitivity may be good serum biomarkers for gastric cancer. Later studies in a larger population group are necessary to confirm this finding. Next, we identified the 4268Da marker as the fragment of Isoform 1 of Putative gonadotropin-releasing hormone II receptor (GNRHR2) by MS/MS.

GNRHR2 is receptor for gonadotropin releasing hormone II (GnRH II), and belongs to the G-protein coupled receptor 1 family. It mediates its action by association with G proteins that activate a phosphatidylinositol-calcium secondary messenger system. In non-hominoid primates and non-mammalian vertebrates, GnRHR2 encodes a seven-transmembrane G-protein coupled receptor. However, in humans, the N-terminus of the predicted protein contains a frame shift and premature stop codon. In humans, GnRHR2 transcription occurs but whether the gene produces a functional C-terminal multi-transmembrane protein is currently unresolved. Alternative splice variants have been reported. An untranscribed pseudogene of GnRHR2 is also located on chromosome 14. GnRH II receptor mRNA was ubiquitously expressed. GnRH II is involved in the suppression of cell growth in tumor cell lines (32). The expression of functional type II GnRH receptor transcripts in human sperm could be part of the existing network of intratesticular or neuroendocrine hormonal regulation governing spermatogenesis. The physiological roles played by GnRH II and its receptor are unknown, so research is underway to more clearly define the function(s) of GnRH II and its receptor. Whatever answers are forthcoming from these studies, we predict that GnRHR II may play an important role in gastric tumorigenesis.

To the best of our knowledge, this is the first description of a serum diagnostic model for gastric cancer established using affinity bead and MALDI-TOF-MS based proteomic techniques. Our model appears to have excellent sensitivity and a specificity, which is higher than other gastric cancer markers available presently. The proteins we have identified are potential serum biomarkers for the diagnosis of gastric cancer however identification and functional analysis is important: In addition to confirming their utility as markers, their function may provide new insights into tumor development and environmental responsiveness, which could eventually be translated into new diagnostic and prognostic insights for the clinician.

In summary, we have described a novel serum proteomic technique using magnetic affinity beads to purify proteins prior to MALDI-TOF-MS analysis. This technique allowed for identification of specific peaks within the protein spectra for comparison with control samples. The initial data is promising but a larger collected sample set, possibly from a multi-centre trial, is necessary to validate and identify the differentially expressed proteins we identified.

## 6. ACKNOWLEDGEMENTS

This work was supported by two grants from the National Natural Science Foundation of China (No.30872477 and No.30901729). Some technological developments used were supported by the Shanghai Leading Academic Discipline Project (Number:S30204) and the National High Tech Program(Nunber:2006AA02A402, 2006AA02A301).

## 7. REFERENCES

1. Liotta, L. A.; Ferrari, M.; and Petricoin, E. Clinical proteomics: Written in blood. *Nature* 425:905-12. (2003)

2. Anderson, N. L.;and Anderson, N. G. Proteome and proteomics: New technologies, new concepts and new words. *Electrophoresis* 19**:**1853-61. (1998)

3. Petricoin, E. F.; Ardekani, A. M.; Hitt, B. A.; Levine. P. J. Fusaro, V. A.; Steinberg, S. M.; Mills, G. B.; Simone, C.; Fishman, D. A.; Kohn, E. C.; Liotta, L. A. Use of

proteomic patterns in; serum to identify ovarian cancer. *Lancet* 359**:**572-7. (2002)

4. Hilario, M.; Kalousis, A.; Muller, M., and Pellegrini, C. Machine learning approaches to lung cancer prediction from mass spectra. *Proteomics* 3:1716-9. (2003)

5. Kozak, K. R.; Amneus, M. W.; Pusey, S. M.; Su, F.; Luong, M. N.; Luong, S. A.; Reddy, S. T.; Farias-Eisner, R. Identification of biomarkers for ovarian cancer using strong anion-exchange ProteinChips: Potential use in diagnosis and prognosis. *Proc. Natl. Acad. Sci. USA* 100:12343-8. (2003)

6. Parkin, D. M. Global cancer statistics in the year 2000. *The Lancet Oncology* 2:533-43. (2001)

7. He, Q. Y.; Cheung, Y. H.; Leung, S. Y.; Yuen, S. T.; Chu, K. M.; Chiu, J. F. Diverse proteomic alterations in gastric adenocarcinoma. *Proteomics* 4:3276-87. (2004)

8. Liu, W. ; Liu, B. ; Xin, L. ; Zhang, Y, ; Chen, X. ; Zhu, Z. ; Lin, Y. Down-regulated expression of complement factor I: A potential suppressive protein for gastric cancer identified by serum proteome analysis. *Clinica chimica acta; International Journal of Clinical Chemistry* 377:119-26. (2007)

9. Hao, Y. ; Yu, Y. ; Wang, L. ; Yan, M. ; Ji, J. ; Qu, Y. ; Zhang, J. ; Liu, B. ; Zhu, Z. IPO-38 is identified as a novel serum biomarker of gastric cancer based on clinical proteomics technology. *Journal of Proteome Research* 7:3668-77. (2008)

10. Ren, H.; Du, N.; Liu, G.; Hu, H. T.; Tian, W.; Deng, Z. P.; Shi, J. S. Analysis of variabilities of serum proteomic spectra in patients with gastric cancer before and after operation. *World J Gastroenterol* 12:2789-92. (2006)

11. Poon, T. C.; Sung, J. J.; Chow, S. M.; Ng, E. K.; Yu, A. C.; Chu, E. S.; Hui, A. M.; Leung, W. K. Diagnosis of gastric cancer by serum proteomic fingerprinting. *Gastroenterology* 130:1858-64. (2006)

12. Ketterlinus, R.; Hsieh, S. Y.; Teng, S. H.; Lee, H.; Pusch, W. Fishing for biomarkers: analyzing mass spectrometry data with the new ClinProTools software. *BioTechniques* Suppl: 37-40. (2005)

13. Villanueva, J.; Shaffer, D. R.; Philip, J.; Chaparro, C. A.; Erdjument-Bromage, H.; Olshen, A. B.; Fleisher, M.; Lilja, H.; Brogi, E.; Boyd, J.; Sanchez-Carbayo, M.; Holland, E. C.; Cordon-Cardo, C.; Scher, H. I.; Tempst, P. Differential exoprotease activities confer tumor-specific serum peptidome patterns. *Journal of Clinical Investigation* 116:271-84. (2006)

14. Chang, J. T.; Chen, L. C.; Wei, S. Y.; Chen, Y. J.; Wang, H. M.; Liao, C. T.; Chen, I. H.; Cheng, A. J. Increase diagnostic efficacy by combined use of fingerprint markers in mass spectrometry--plasma peptidomes from nasopharyngeal cancer patients for example. *Clinical Biochemistry* 39:1144-51. (2006)

15. Swets, J. A. Measuring the accuracy of diagnostic systems. *Science* 240:1285-93. (1988)

16. Nakajima, T. Gastric cancer treatment guidelines in Japan. *Gastric Cancer* 5:1-5. (2002)

17. Nakane, Y. OS. ; Akehira, K. ; Akehira, K. ; Boku, T. ; Okusa, T. ; Tanaka, K. ; Hioki, K. Correlation of preoperative carcinoembryonic antigen levels and prognosis of gastric cancer patients. *Cancer* 73:2703-8. (1994)

18. Pectasides, D. MA. ; Kostopoulou, M. ; Papadopoulou, M. ; Triantafillis, D. ; Varthalitis, J. ; Dimitriades, M. ; Athanassiou, A. CEA, CA 19-9, and CA-50 in monitoring gastric carcinoma. *Am J Clin Oncol* 20:348-53. (1997)

19. Kozak, K. R.; Amneus, M. W.; Pusey, S. M.; Su, F.; Luong, M. N.; Luong, S. A.; Reddy, S. T.; Farias-Eisner, R. Identification of biomarkers for ovarian cancer using strong anion-exchange ProteinChips: potential use in diagnosis and prognosis. *Proc Natl Acad Sci U S A* 100:12343–8. (2003)

20. Petricoin, E. F.; Ardekani, A. M.; Hitt, B. A.; Levine, P. J.; Fusaro, V. A.; Steinberg, S. M.; Mills, G. B.; Simone, C.; Fishman, D. A.; Kohn, E. C.; Liotta, L. A. Use of proteomic patterns in serum to identify ovarian cancer. *Lancet* 359:572–7. (2002)

21. Ebert, M. P. ; Meuer, J. ; Wiemer, J. C. ; Schulz, H. U. ; Reymond, M. A. ; Traugott, U. ; Malfertheiner, P. ; Röcken, C. Identification of gastric cancer patients by serum protein profiling. *Journal of Proteome Research* 3:1261-6. (2004)

22. Ransohoff, D. F. Rules of evidence for cancer molecular-marker discovery and validation. *Nature Reviews* 4:309-14. (2004)

23. Qiu, F.; Liu, H. Y.; Zhang, X. J.; Tian, Y. P. Optimization of magnetic beads for MALDI-TOF MS analysis. *Frontiers in Bioscience* 14:3712-23. (2009)

24. Qiu, F.; Liu, H. Y.; Dong, Z. N.; Feng, Y. J.; Zhang, X. J.; Tian, Y. P. Searching for Potential Ovarian Cancer Biomarkers with Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry. *Am. J. Biomed. Sci.* 1:80-90. (2009)

25. Kojima, K.; Asmellash, S.; Klug, C. A.; Grizzle, W. E.; Mobley, J. A.; Christein, J. D. Applying proteomic-based biomarker tools for the accurate diagnosis of pancreatic cancer. *J Gastrointest Surg* 12:1683-90. (2008)

26. Dekker, L. J.; Boogerd, W.; Stockhammer, G.; Dalebout, J. C.; Siccama, I.; Zheng, P.; Bonfrer, J. M.; Verschuuren, J. J.; Jenster, G.; Verbeek, M.; Luider, T. M.;

Smitt, P. A. MALDI-TOF mass spectrometry analysis of cerebrospinal fluid tryptic peptide profiles to diagnose leptomeningeal metastases in patients with breast cancer. *Mol Cell Proteomics* 4:1341-9. (2005)

27. Ebert, M. P.; Niemeyer, D.; Deininger, S. O.; Wex, T.; Knippig, C.; Hoffmann, J.; Sauer, J.; Albrecht, W.; Malfertheiner, P.; Röcken, C. Identification and confirmation of increased fibrinopeptide a serum protein levels in gastric cancer sera by magnet bead assisted MALDI-TOF mass spectrometry. *Journal of Proteome Research* 5:2152-8. (2006)

28. Lim, J. Y.; Cho, J. Y.; Paik, Y. H.; Chang, Y. S.; Kim, H. G. Diagnostic application of serum proteomic patterns in gastric cancer patients by ProteinChip surface-enhanced laser desorption/ionization time-of-flight mass spectrometry. *International Journal of Biological Markers* 22:281-6. (2007)

29. Su, Y. ; Shen, J, ; Qian, H. ; Ma, H. ; Ji, J. ; Ma, H. ; Ma, L. ; Zhang, W. ; Meng, L. ; Li, Z. ; Wu, J. ; Jin, G. ; Zhang, J. ; Shou, C. Diagnosis of gastric cancer using decision tree classification of mass spectral data. *Cancer Science* 98:37-43. (2007)

30. Liang, Y.; Fang, M.; Li, J.; Liu, C. B.; Rudd, J. A.; Kung, H. F.; Yew, D. T. Serum proteomic patterns for gastric lesions as revealed by SELDI mass spectrometry. *Experimental and Molecular Pathology* 81:176-80. (2006)

31. Qian, H. G.; Shen, J.; Ma, H.; Ma, H. C.; Su, Y. H.; Hao, C. Y.; Xing, B. C.; Huang, X. F.; Shou, C. C. Preliminary study on proteomics of gastric carcinoma and its clinical significance. *World J Gastroenterol* 11:6249-53. (2005)

32. Emons, G.; Muller, V.; Ortmann, O.; Schulz, K-D. Effects of LHRH-analogues on mitogenic signal transduction in cancer cells. *J Steroid Biochem Mol Biol* 65:199–206. (1998)

**Key Words:** Gastric cancer, Serum, Diagnostic model, Magnetic beads, CLINPROT

**Send correspondence to: Qiumeng Yang**, Department of Surgery, Shanghai Institute of Digestive Surgery, Ruijin Hospital, Shanghai Jiao Tong University, 197 Ruijin Road II, Shanghai 200025 PR China, Fax: 86-21-64670644, Tel: 86-21-64370045-666509, E-mail: yangqiumeng@hotmail.com