Int J Clin Exp Med 2017;10(1):929-936
www.ijcem.com /ISSN:1940-5901/IJCEM0040052

Original Article

Detection and significance of small-cell lung cancer serum protein markers using MALDI-TOF-MS

Zhihua Li[1,2], Chuanhao Tang[1], Xiaoyan Li[1], Jianjie Li[1], Weixia Wang[1], Haifeng Qin[1], Bin Xu[3], Jian Chen[4], Hongjun Gao[1], Kun He[3], Xiaoqing Liu[1]

[1]Department of Pulmonary Oncology, 307 Hospital, PLA, Beijing 100071, China; [2]Department of Oncology, General Hospital of The PLA Rocket Force, Beijing 100088, China; [3]National Center of Biomedical Analysis, Beijing 100850, China; [4]Affiliated Hospital of Institute of Aviation Medicine, Air Force, Beijing 100089, China

Received September 14, 2016; Accepted November 10, 2016; Epub January 15, 2017; Published January 30, 2017

Abstract: Early diagnosis is vital to improving the survival rate of patients with small-cell lung cancer (SCLC). Considering recent applications of mass spectrometry (MS) to cancer research, this study aimed to identify reliable biomarkers for an early and accurate diagnosis of SCLC. A total of 160 serum samples, including 80 from SCLC patients and 80 from healthy controls, were analysed using the ClinPro system combined with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). Using the ClinPro software and a genetic algorithm analysis, a panel of serum markers that efficiently predicted the patients who had SCLC was selected. A supervised neural network algorithm model that included five peptides/proteins was developed from the training group to distinguish SCLC patients from healthy individuals. In this study, we identified peptide/protein differences in serum samples from SCLC patients and healthy individuals and established a serum peptide-based classification of SCLC patients with high sensitivity and specificity using an MALDI-TOF-MS system.

Keywords: Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, serum peptide profiles, small-cell lung cancer

Introduction

Lung carcinoma, also known as primary bronchogenic carcinoma of the lung, is among the most common types of cancer worldwide [1]. Lung cancer is usually divided into non-small-cell lung cancer (NSCLC) and small-cell lung cancer (SCLC), and the latter accounts for about ~15-20% of lung cancer cases diagnosed annually and 25% of the deaths caused by lung cancer [2, 3]. Because SCLC is aggressive and characterized by rapid growth, early metastasis, and relapse, most SCLC patients are not cured by surgical procedures and have a poor prognosis. The median survival of SCLC patients with limited and extensive disease is 12-24 and 7-11 months, respectively, and less than 5% of SCLC patients can survive more than 5 years [4].

Therefore, early diagnosis is important to improve the treatment response rate and survival. However, many patients undergo a delayed examination and further treatment because they exhibit no specific symptoms. Currently, the gold standard for the diagnosis of SCLC depends on the cytological pathology, which results in many patients delaying treatment because of concerns about the invasiveness of the diagnostic procedure. Thus, it is urgently necessary to discover reliable biomarkers that can lead to an early and accurate diagnosis of SCLC. Recent advances in proteomics and related technologies makes it possible to detect biomarkers for the early diagnosis of cancer and provide powerful tools to describe in detail the pathogenesis of tumours. Mass spectrometry, the core technology of proteomics, is widely used in screening for new potential protein biomarkers for various types of tumours [5-10].

In our present study, we used a novel proteomic technology, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) coupled with the ClinPro system, which is a relatively new proteomics tech-

nology that is considered to be one of the most powerful tools for differential expression profiling, to search for peptide and/or protein biomarkers. Coupled with the ClinPro™ system, we applied peptide mass fingerprinting using MALDI-TOF-MS to analyse the serum of SCLC patients and healthy controls and detected the differences in serum peptides/proteins between the two groups. We developed a serum proteomic diagnostic classification for SCLC and tested it on an independent validation group to detect potential diagnosis-related biomarkers of SCLC, which may serve as a supplement to traditional imaging and pathological diagnostic methods.

Patients and methods

Study population and specimens

A total of 80 pre-treatment serum samples were obtained from the Department of Lung Cancer of the Affiliated Hospital of the Academy of Military Medical Science between October 2012 and October 2014. Inclusion criteria were as follows: pathologically confirmed SCLC, an Eastern Cooperative Oncology Group (ECOG) performance status of 0 or 1, older than 18 years old, and without severe underlying diseases (heart, liver and kidney).

Over the corresponding period, 80 serum samples were collected from healthy individuals who underwent a physical examination at the clinic. These healthy individuals were required to meet the following criteria: older than 18 years old, and without pulmonary nodules, pneumonia, and tuberculosis or other abnormalities. This study was performed under protocols approved by the local institutional review boards. All patients provided written informed consent to participate in this study and provided permission for the use of their blood samples. Smoking status was based on records at the first visit of patients to the clinic. Having smoked more than 100 cigarettes in a lifetime was used to define smokers.

Whole blood samples (5 ml) were collected before patients received the first-line therapy in a test tube, and blood was allowed to clot at room temperature for 1 h. After centrifugation at 3000 rpm for 10 min at 4°C, serum was divided into aliquots and immediately stored at -80°C until later use.

Peptidome isolation

We used weak cation exchange magnetic beads (MB-WCX, National Center of Biomedical Analysis, China) to fractionate serum samples following the standard protocol recommended by the manufacturer (Bruker Daltomik GmbH). *Step 1, Binding.* We mixed 20 µl binding solution (National Center of Biomedical Analysis, China), 5 µl MB-WCX beads that had been washed three times in 50 µl binding solution and 5 µl serum in a polymerase chain reaction tube, which was incubated for 10 min at room temperature. *Step 2, Washing.* We separated the unbound solution with a magnetic bead separation device, and beads were washed three times with 100 µl washing solution (National Center of Biomedical Analysis, China). *Step 3, Elution.* Bound proteins/peptides were eluted from the magnetic beads with 20 µl eluting solution (National Center of Biomedical Analysis, China) for MALDI-TOF-MS analysis.

MALDI-TOF-MS analysis

For MALDI-TOF-MS analysis, 1 µl peptide eluate was mixed 1:1 (v/v) with a matrix solution that consisted of saturated α-cyano-4-hydroxy-cinnamic acid (α-HCCA, Bruker Daltonics, Germany) in 50% acetonitrile (ACN, Sigma-Aldrich, USA) and 0.1% trifluoroacetic acid (TFA, Sigma-Aldrich, USA) was spotted onto the sample anchor spots of an AnchorChip 600/384 target plate (Bruker Daltonics, Germany), which was allowed to air-dry at room temperature to let the matrix crystallize. ClinPro Peptide Calibration Standard I (Bruker Daltonics, Germany), a commercially available mixture of protein/peptide calibrators that consisted of angiotensin II (m/z 1,047.19), angiotensin I (m/z 1,297.49), substance P (m/z 1,348.64), bombesin (m/z 1,620.86), ACTH clip 1-17 (m/z 2,094.43), ACTH clip 18-39 (m/z 2,466.48), and somatostatin (m/z 3,149.57), was mixed 1:1 (v/v) with matrix solution, and 0.5 ml of the mixture was deposited on the calibrant anchor spots of an AnchorChip target plate for instrument calibration.

Mass spectrometry analyses were performed on an Ultraflex III MALDI-TOF-MS (Bruker Daltonics, Germany). The operating conditions were as follows: linear positive ion mode; repetition rate, 200 Hz; ion source voltages, 25 and 23.50 kV; lens voltage, 6.5 kV; and pulsed ion extrac-

Table 1. Clinical and disease characteristics of study participants

Characteristics	Training group (n=100)			Validation group (n=60)		
	SCLC group I (n=50)	Healthy group I (n=50)	*P*	SCLC group II (n=30)	Healthy group II (n=30)	*P*
Age, y			0.372			0.465
Range	19-76	25-77		24-74	25-72	
Median	57	56		55	59	
Sex, No. (%)			0.677			0.793
Male	33 (66%)	31 (62%)		18 (60%)	17 (56.7%)	
Female	17 (34%)	19 (38%)		12 (40%)	13 (43.3%)	
Smoking history, No. (%)			0.295			0.795
Smoker	35 (70%)	30 (60%)		17 (56.7%)	16 (53.3%)	
Never smoker	15 (30%)	20 (40%)		13 (43.3%)	14 (46.7%)	
Disease stage, No. (%)			NA			NA
Limited	9 (18%)	-		6 (20%)	-	
Extensive	41 (82%)	-		24 (80%)	-	
Performance status, No. (%)			0.056			0.492
ECOG PS 0-1	45 (90%)	50 (100%)		28 (83.3%)	30 (100%)	
ECOG PS 2	5 (10%)	0 (0%)		2 (16.7%)	0 (0%)	

NA: not assessed; ECOG PS: Eastern Cooperative Oncology Group Performance Status.

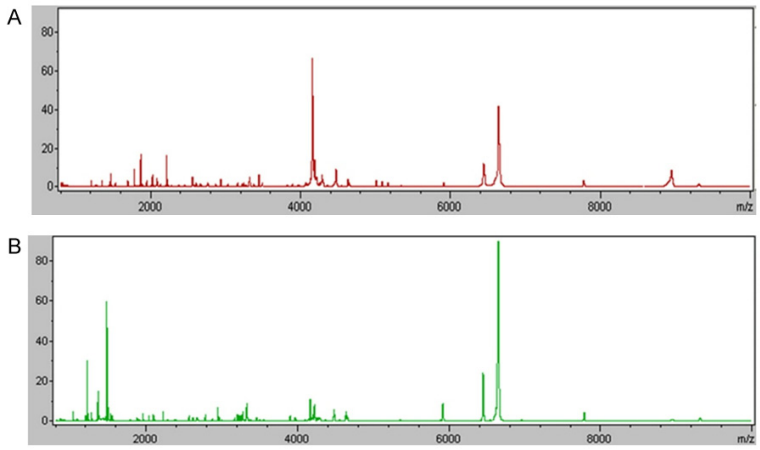

Figure 1. The average spectra of the training set displayed by ClinPro Tools software. A*:* Average spectra for SCLC patients in the training group. B*:* Average spectra of healthy individuals in the training group.

tion time, 100 ns. For matrix suppression, we used a high gating factor with signal suppression of up to 300 m/z. For each spectrum, 3000 shots were manually acquired from six random positions over the surface of each spot (i.e., 500 shots per position). Data acquisition was carried out at 43% of the maximum laser energy. Each spectrum was externally calibrated. Peaks in the m/z range of 800-10,000 Da were recorded using FlexControl acquisition software v3.4 (Bruker Daltonics, Germany).

Quantification of serum neuron-specific enolase (NSE) and ProGRP

We measured the two SCLC markers in the serum, NSE and ProGRP, in all 30 SCLC patients and 30 healthy individuals in the blinded test set using an electrochemiluminescent immunoassay following the standard protocol recommended by the manufacturer.

Bioinformatics

Spectral processing: ClinPro Tools software v2.1 (Bruker Daltonics, Germany) was used to automatically process MALDI-TOFMS spectra data using data preparation settings according to the following standard workflow. Each raw spectrum was normalized to its total ion current. All spectra were recalibrated using the prominent, common m/z values. Next, baseline subtraction, smoothing, and peak detection were performed, and the peak areas for each spectrum were calculated. The signal-to-noise ratio was set at 5 for peak detection. Peak areas were calculated using zero level integra-

Table 2. The 21 differential peaks in serum from the SCLC patients and the healthy individuals in the training group

M/z	Peak areas of the healthy group I (X±S)	Peak areas of the SCLC group I (X±S)	Peak areas in SCLC patients compared with the healthy individuals
1207.99	178.99±82.75	25.02±19.1	↓
1021.55	33.18±11.64	8.08±2.7	↓
1190.27	29.66±9.95	10.82±3.78	↓
1221.66	28.99±11.6	9.78±4.37	↓
1467.31	382.01±229.91	52.72±50.27	↓
8944.33	50.44±24.55	409.17±305.51	↑
1481.1	59.89±36.54	13.93±9.51	↓
1351.88	93.13±42.33	27.4±18.01	↓
1264.49	34.9±12.21	12.6±7.13	↓
4068.72	22.38±4.47	53.36±28.29	↑
4298.33	14.24±3.51	45.26±25.99	↑
1520.33	34.52±8.75	15.97±7.99	↓
3193.18	39.54±13.85	15.78±8.19	↓
4112.1	9.26±2.59	25.55±15.37	↑
1365.48	20.49±5.9	10.57±4.31	↓
3263.64	58.08±22.41	20.81±12.84	↓
9437.31	10.67±5.66	5.23±2.7	↓
4137.6	30.37±7.79	104.04±66.5	↑
1078.53	22.63±5.59	12.24±5.08	↓
1866.81	21.74±11.09	130.93±121.92	↑
3815.93	15.89±2.78	28.46±9.99	↑

↑: Peak areas in SCLC patients is bigger compared with the healthy individuals; ↓: Peak areas in SCLC patients is smaller compared with the healthy individuals.

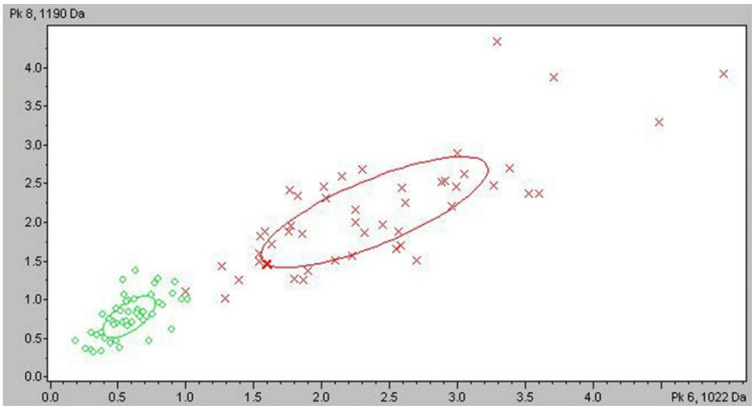

Figure 2. 2D peak distribution of peptides with m/z 1021.55 (x-axis) and 1190.27 (y-axis) between SCLC patients (red circles) and healthy individuals (green crosses). The discriminating features of the two selected peptides were generated using ClinPro Tools bioinformatics software. Values represent the peptide abundance ratio; these values were significantly different between the two groups. Ellipses represent the standard deviation of the class average of peak areas/intensities.

tion type. Spectra were also "top hat" baseline subtracted from the minimum baseline width set to 10%, and then were smoothed and processed in the 800-10,000 Da range.

Establishment of a training and classification model: Spectra from the training groups were used to build a classification model. Differential peptides peaks between the 50 SCLC patients and 50 healthy individuals were selected using peak areas that exhibited statistically significant differences. The built-in mathematical model's Genetic algorithm (GA), Supervised Neural Network (SNN) and quick classifier algorithm (QC) were used to select each peptide peak and classification models were setup using ClinPro Tools 2.1 software to determine the optimal separation planes between samples from the two training groups. After each model was generated, a random cross-validation process was performed with the software, and the percent to omit and the number of interactions were set at 20 and 10, respectively.

To determine the accuracy of the class prediction model, the software offers cross validation and recognition capability. Cross validation is a measure of the reliability of a calculated model and can be used to predict how a model will behave in the future. This method is used to evaluate the performance of a classifier for a given data set under a given parameterization. Recognition capability describes the performance of an algorithm, i.e., the proper classification of a given data set.

Blind test of the classification model: The separated samples of the validation groups from the 30 SCLC patients and 30 healthy volunteers were used

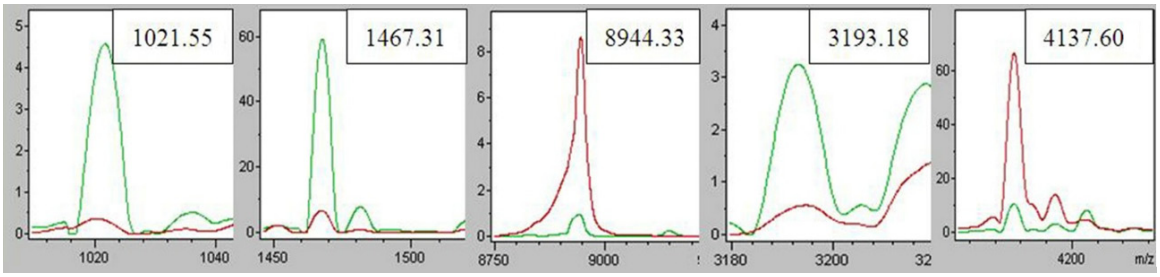

Figure 3. ClinPro Tools image showing the average intensity, in arbitrary units, of five peptides that represent the classifier in SCLC patients and healthy individuals.

to show the efficacy and accuracy of the classification model. Validation was performed in a blinded manner, as that MALDI-MS analysis was performed and classifications were labelled before clinical outcome data were made available to the investigators. For each sample from the validation groups, a corresponding spectrum was presented to the selected classification model. Then, the software returned a result that was compared with the actual pathological diagnosis.

Statistical analysis

Comparisons of the clinical characteristics and the positive rate between different groups were made using the x^2 or Fisher's exact test. Statistical analyses were performed using the SPSS software v19.0 (SPSS Inc., USA). A *p*-value less than 0.05 was considered to indicate a statistically significant difference. Comparisons of the area under the peptide peaks between different groups were made using the t-test with ClinPro Tools software (version 2.1).

Results

Patient characteristics

A total of 80 SCLC patients and 80 healthy individuals who met our enrolment criteria were enrolled in this study. Additionally, 50 SCLC patients (termed the SCLC group I) and 50 healthy individuals (termed the healthy group I) were randomly selected from the two groups to form a training group; the remaining 30 SCLC patients (termed the SCLC group II) and 30 healthy individuals (termed the healthy group II) formed the validation group. The clinical and disease characteristics of these groups are listed in **Table 1**. Patients were balanced between the training group and validation group. In the training group, there were no significant differences detected between the SCLC patients and healthy individuals for age, sex, smoking history or performance status.

Differences in serum peaks between SCLC patients and healthy individuals in the training group

The training group included 50 SCLC patients (termed SCLC group I) and 50 healthy individuals (termed healthy group I) and the total average peptide spectrum of the two groups was analysed using ClinPro Tools software analysis (**Figure 1**). A total of 109 peptide peaks was identified in the spectra of the training group data set that was generated by MALDI-TOF-MS; 21 peaks were significantly different ($P< 0.000001$, $AUC\geq0.9$) between the two groups (**Table 2**). A total of 14 signals exhibited a lower peak area, and 7 signals exhibited a higher peak area in SCLC patients compared with healthy individuals. Peptide peaks with m/z 1021.55 and 1190.27 exhibited the greatest difference in peak areas and were plotted in 2D peak distribution view (**Figure 2**).

Establishment of a classification model

A total of three algorithms-GA (optimized by adjusting the number of neighbours for a k-nearest neighbour classification), SNN and QC-were applied for classification model construction using spectral data from the training group that was generated by MALDI-TOF-MS. By comparing the recognition capability and cross-validation of the models, we generated the optimal model-adopted SNN algorithm. This model was composed of five peptide peaks with m/z 1021.55, 1467.31, 8944.33, 3139.18 and 4137.6, and exhibited the best efficiency in separating samples from SCLC patients versus healthy individuals, with a recognition capability of 98.96% and a cross-validation capability of 95.84% (**Figure 3**).

Table 3. Blind test results of the model in the validation group

Pathological diagnosis	MALDI-TOF-MS classification		Total number	Sensitivity (%)	Specificity (%)	Accuracy (%)
	"SCLC patients"	"Healthy individuals"				
SCLC patients	28	3	30	96.7%	90.0%	91.7%
Healthy individuals	3	27	30			

Table 4. Sensitivities and specificities of the classification model, NSE and ProGRP

Items	Model	NSE	ProGRP	Combination
Sensitivity (%)	96.7 (28/30)	53.3 (16/30)[a]	63.3 (19/30)[a]	80.0 (24/30)[b]
Specificity (%)	90.0 (27/30)	73.3 (20/30)[a]	80.0 (24/30)[b]	60.0 (18/30)[a]

Model: classification model; Combination: combined use of NSE and ProGRP. [a]$P<0.05$ compared with the model; [b]$P>0.05$ compared with the model.

Blinded test of the classifier in the validation group

The classifier was then validated using an independent validation group of 30 SCLC patients and 30 healthy individuals in a blinded test (**Table 3**). Among the 30 samples from SCLC patients, 28 (96.7%) were labelled as "SCLC patients" by the serum proteomics classifier, while among the 30 samples from healthy individuals, 27 (90.0%) were labelled as "healthy individuals", achieving an overall accuracy of 91.7%, with a sensitivity of 96.1% and specificity of 90.0%, which indicated robust consistency between the pathological diagnosis and serum proteomics classifier.

Comparisons with serum NSE and ProGRP

Table 4 shows results of the sensitivities and NSE and pro-gastrin-releasing peptide (ProGRP). We compared the diagnostic capacities of the classification model with NSE and ProGRP, both alone and combined.

Discussion

Early diagnosis appears to be the most appropriate tool for reducing disease-related mortality in most malignant tumours, especially for the treatment of SCLC. The survival rate and time of those patients with limited stage SCLC are obviously better than those of patients with extensive stage disease. However, the development of diagnostic techniques has not yet improved the rate of early diagnosis. Biological mass spectrometry (MS), as the core technology of proteomics, may represent a novel technology for detecting SCLC biomarkers. Preliminary studies established that these techniques may provide a novel non-invasive way to diagnose malignant cancer; moreover, these techniques may have additional value as prognostic tools [11-15]. The most commonly used instruments of biological mass spectrometry technology include Surface-Enhanced Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (SELDI-TOF-MS), Electrospray Ionization-Mass Spectrometry (ESI-MS) and MALDI-TOF-MS. Mass spectrometry instrumentation and analysis tools have continued to rapidly evolve and improve our ability to detect less-abundant serum proteins. Many researchers have attempted to apply them to the diagnosis of lung cancer. Han [16] and Simsek et al [17] applied SELDI-TOF-MS technology to detect proteome profiles that were specific to SCLC, NSCLC and non-malignant disease, for which the sensitivity and specificity were 75% to 85%. Hocker et al [18] reported that a proteomic panel of ESI-MS tools yielded a sensitivity of 84% and accuracy of 80% in distinguishing NSCLC patients from healthy controls. These studies indicated SELDI-TOF-MS and ESI-MS could be used to effectively develop diagnostic criteria to distinguish SCLC and NSCLC.

Compared with other modes of MS instrumentation, MALDI-TOF-MS has the advantages of high sensitivity and resistance to salt and pollution. Additionally, it has a high distinguishability with an accuracy of 10 ppm when 2,000 Da small molecule peptides are assayed. In this present study, we utilized the ClinPro™ system developed by the Bruker Daltonics Company (Germany). The system includes peptidome isolation with magnetic beads, MALDI-TOF-MS and built-in analysis software. It is stable and reliable with high sensitivity and repeatability. The

liquid magnetic beads can combine low abundance proteins or peptides. Additionally, the analysis process is rapid and only requires a small amount of sample for standardization and can operate in a high throughput manner [19].

The ClinPro system provides optimal reproducibility and is suitable for automated peptide profiling. It has the capability to simultaneously identify potential biomarker proteins. In this present study, we attempted to build a serum peptide classification model to distinguish SCLC patients from healthy individuals using MALDI-TOF-MS along with the ClinPro system. To the best of our knowledge, this study is one of a few to screen SCLC-related peptides in serum using the ClinPro system. We examined 50 serum samples from SCLC patients and 50 samples from healthy individuals with this system, and the classification model was constructed to distinguish SCLC cases from healthy individuals using 5 peptide peaks at 1021.55, 1467.31, 8944.33, 3139.18 and 4137.6 Da as the marker pattern. When the model was tested using a blinded test set, it yielded a sensitivity of 96.7% (28/30), specificity of 90.0% (27/30), and accuracy of 91.7% (55/60). For comparison, NSE and ProGRP were also measured in our study. Although there were no significant differences between the sensitivity of the classification model and the combination of NSE and ProGRP, or between the specificities of the model and ProGRP, the sensitivity of the model was significantly higher than that of NSE and ProGRP. Moreover, the specificity achieved by this model was significantly higher than that of NSE or the combination. These findings indicate that the classification model built using the MALDI-TOF-MS system is superior to that of NSE and ProGRP, or a combination, to distinguish SCLC patients from healthy controls.

Compared with tissues, sputum and pleural effusions, serum can be readily obtained from patients and has the advantages of being non-invasive, showing repeatability and having simple sample preparation requirements. We can obtain serum samples at any time if there is a need for diagnosis during the treatment process. The weak cation exchange magnetic beads that we used in this study combined many types of low abundance proteins/peptides and avoided peptide losses during removal of high abundance proteins. This resulted in high sensitivity and specificity for the system [20, 21]. In this present study, we utilized comparative proteomics to compare serum samples from different groups and established a diagnostic classification model for SCLC based on a panel of peptides. When a tumour exists in the body, the proteins/peptides in the serum will change as a consequence of changes in gene expression, metabolism and the internal environment. Changes of serum proteins/peptides can be identified by the classification model based on our peptide panel, so we can rapidly and reliably distinguish SCLC patients from healthy individuals. Some researchers believe that measuring panels of peptide markers may be more sensitive and specific than conventional biomarker approaches because they 'record' the cellular and extracellular enzymatic events that occur at the level of the cancer-tissue microenvironment [22, 23].

In conclusion, we directly profiled peptide patterns from the MB-WCX-purified serum samples with MALDI-TOF-MS, and constructed a peptide model to differentiate SCLC patients from healthy volunteers with high sensitivity and specificity, even though the study had some constraints. The next step in our research will be to extend our study population to different centres to confirm the utility of our currently identified peptides for SCLC diagnosis. Additionally, we will employ other proteomic technologies and bioinformatics approaches to isolate and identify biomarkers of interest and study their biological roles in SCLC pathogenesis.

Acknowledgements

The present study was funded by the Chinese National Instrumentation Program (Beijing, China, grant no. 2011YQI70067).

Disclosure of conflict of interest

None.

Address correspondence to: Xiaoqing Liu, Department of Pulmonary Oncology, 307 Hospital, PLA, Beijing 100071, China. Tel: +86 01066947161; E-mail: liuxq@medmail.com

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