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Serum Proteomic Profiling of Lung Adenocarcinoma with Brain Metastasis Based on Matrix-Assisted Laser Ionization Time of Flight Mass Spectrometry Analysis

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Abstract

Background: Once patients presented with brain metastasis, the prognosis is poor with shortened survival of up to 6 months, and therefore early recognition of brain metastasis may be beneficial to outcomes of patients. The present work focused on serum proteomic biomarkers that represent the status of lung adenocarcinoma especially for patients with brain metastasis. **Methods:** 100 serum samples including 25 from lung adenocarcinoma patients with brain metastasis , 25 from lung adenocarcinoma patients without metastasis and 50 from healthy controls were analyzed using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). ClinProt software and binary logistic regression method were used to develop classification model using MS spectral data. Univariate and Cox multivariate analysis were also performed to evaluate the potential prognostic value of serum peptides. **Results:** A series of 14 significant short peptides was detected in serum of lung adenocarcinoma patients as compared with healthy controls. Of these, a panel consisting of peptides m/z 1969.9 and 2213.4 Da had the highest diagnostic value for discriminating lung adenocarcinoma from healthy controls with a sensitivity of 91.7% and a specificity of 79.2%. The panel consisting of peptides m/z 1781.5 and 1984.7 Da had the highest diagnostic value for discriminating advanced lung adenocarcinoma with brain metastasis from patients without metastasis with a sensitivity of 84.6% and a specificity of 79.2%. Univariate and Cox multivariate analysis disclosed that peptide m/z 1969.9 Da remained an independent predictors for lung adenocarcinoma patients while peptide m/z 1984.7 Da shows favourable value on pre-warning lung adenocarcinoma patients

with brain metastasis after chemotherapy. **Conclusion:** We have completed a preliminary study to describe the serum proteomic profile of lung adenocarcinoma patients with brain metastasis, and our proteomic models may improve the diagnosis and prognosis of lung adenocarcinoma patients and helps us to better understand the pathogenesis of disease process of brain metastasis.

Keywords: Classification model, prognostic value, serum peptides, lung adenocarcinoma, brain metastasis.

1. Introduction

Metastatic spread to brain are common for patients with Non-small cell lung cancer (NSCLC) (8). At initial diagnosis, 20% of NSCLC patients have brain metastasis (BM), approximately 40-50% develop BM during the course of treatment (16,19,24). For NSCLC patients with BM, the primary treatment remains systemic chemotherapy, surgery and radiotherapy, and therefore one might expect this to be a logical choice for patients with BM as well. Some patients with BM respond to the treatment to some degree (1,12). But, several issues have limited the application of chemotherapy, among which BM is a major barrier of restricting the use of chemotherapy at some point during the disease (6,22). Early recognition of BM may be beneficial to outcomes. Although several screening techniques, such as analysis of serum tumor markers (23) are recommended, the sensitivity and specificity is relatively low. So it is necessary to explore effective biomarkers for pre-warning the condition of patients.

The usefulness of proteomic analysis for monitoring the condition of cancer patients is now widely recognized (2,5,7,20). In recent years, it has been demonstrated that the serum contains thousands of peptides, most of which are thought to be degraded fragments of large proteins due to the interaction between tumor cells and tumor microenvironment (18,26). And some of these peptides may function as biomarkers for specific physiological and pathological process, with their changes in quantity and quality being correlated with pathological status and thus useful for monitoring the condition of patients. The matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has already been used to detect peptides of low molecular weights at high sensitivity and resolution among cancer patients, which make it a convenient platform for serum peptides profiling(3,4,21).

In the present study, we analyzed serum proteomic pattern in lung adenocarcinoma patients using MALDI-TOF MS. We aim to find potential peptides that represent the status of lung adenocarcinoma especially for patients with BM and establishing classification models for lung adenocarcinoma patients.

2. Materials and methods

2.1. Patients and Sample Collection

Fifty healthy controls and 50 histologically confirmed lung adenocarcinoma patients were enrolled between July 1st, 2012 and June 30th, 2013. The study was performed with the permission of the Ethical Commission of PLA general hospital. Informed consent was also obtained from each patient. In terms of the chemotherapy regimen, all patients received 4~6 cycles of 500 mg/m^2 pemetrexed combined with 75 mg/m^2 cisplatin every 21 days. Brain magnetic resonance imaging (MRI) was performed in all lung adenocarcinoma patients to confirm the presence of BM every two cycles. Serum samples without freeze thawing were stored in small aliquots at −80◦C until used.

2.2. Mass Spectrometry Analysis

Peptides are purified using magnetic bead based weak cation exchange (MB-WCX) on ClinProt platform(Bruker Daltonics Inc., Fremont, CA) (14) according to the manufacturer's instructions. For proteome analysis, we used an Autoflex III MALDI-TOF-MS with the following setting: ion source 1,120 kV; ion source 2,186 kV; lens, 7.6 kV. For each sample, 1600 spectra were acquired (400 laser shots at 8 different spot positions) using linear

positive mode. Spectra were collected automatically using the autoflex Analysis software (Bruker Daltonik) to generate raw data of optimized quality.

2.3. Statistical analysis

All statistical analyses were performed using the SPSS version 20.0 statistical software (SPSS, Inc., Chicago, IL). The ClinProt software 2.0 (Bruker Daltonics Inc., Fremont, CA was used for analysis of all serum sample data derived from either lung adenocarcinoma patients or healthy controls. Statistically significant different quantity of peptides was determined by means of t-tests or Mann–Whitney U-test. The classification models were generated by binary logistic regression. And the progression-free survival (PFS) time was calculated from the day of diagnosis to the day of progression or day of the last follow-up. The cut-off, spearman correlation analysis and AUC (area under the curve) values of selected peptides were carried out using SPSS statistical software as well. The PFS curves were determined using the Kaplan-Meier method and compared between groups using the log-rank test. The independent prognostic factors were conducted by univariate analysis and Cox multivariate analysis. Significance was defined by a p-value<0.05.

3. Results

3.1 Patient characteristics and lung adenocarcinoma-related peptides

The characteristics of the 50 lung adenocarcinoma patients and 50 healthy controls are shown in Table 1, Table 2. The lung adenocarcinoma patients included 31 males and 29 females whose median age was 51 years (range: 27–78 years). Almost two-fifth were current smokers while non-smokes and ex-smokers account for 36% and 26% respectively. A total of 16 patients (32%) were low differentiated, 12 patients (24%) were moderate or well differentiated and the remaining 17 patients (34%) with an unknown status. 25 patients were presented Ⅰ~ⅢA without BM while the other 25 patients presenting in stage ⅢB~Ⅳ with BM.

All the 100 serum samples were detected by MADLI-TOF-MS (mass 1,000~10,000 Da) in combination with MB-WCX magnetic bead. We evaluated proteomic changes in the serum samples of 50 lung adenocarcinoma patients as compared with 50 heathy controls. As shown in table 3, a set of 14 peptide peaks which showed significant differences between lung adenocarcinoma patients and healthy controls were selected by ClinPro software.

Table 1. Baseline characteristics of the 50 lung adenocarcinoma patients.

Clinical Parameters	% of Patients
Age,years	
Median	51
Range	27~78
Gender,n(%)	
Male	31(62%)
Female	19(38%)
Smoking status	
Non-smokers	18(36%)
Ex-smokers	13(26%)
Smokers	19(38%)
Tumor differentiation	
Low	16(32%)
Moderate or well	12(24%)
unkown	17(34%)
Stage,n(%)	
Ⅰ~ⅢA	25(50%)
ⅢB~Ⅳ	25(50%)
Brain Metastasis	
Yes	25(50%)
No	25(50%)
Histology,n(%)	
Adenocarcinoma	50(100%)

Table 2. Basic information of the studied subjects

Histology	No. of patients	Age, mean, median (range)	Sex (female, male)
Healthy control	50	49.81,50 (22,74)	27,23
Lung adenocarcinoma without BM	25	52.56,53 (27,78)	9,16
Lung adenocarcinoma with BM	25	51.13,50(29,73)	10,15

Table 3. Peptide peaks differentially expressed between lung adenocarcinoma patients and healthy controls

Peptides	Normal	Adenocarcinoma	trend	p
1781.8	176.4	469.1	↑	0.000289
1869.1	331.5	814.4	↑	0.000448
1947.9	1946	2270.3	↑	0.004
1969.9	264.6	432	↑	0.00012
2663.8	342.5	856.3	↑	0.008
2865.8	227.3	449	↑	<1e-6
3244.4	590.7	1390.8	↑	0.0000116
4096.2	109.8	272.8	↑	0.006
4214.1	514.1	1203.2	↑	0.000582
5910.2	454.8	886.2	↑	0.016
1984.7	239.2	149.1	↓	0.008
2095.2	1931.3	992.7	↓	0.00000854
2107.1	586	218.6	↓	0.000102
2213.4	4036.9	2088.9	↓	0.0000739

Table 5. Univariate logistic regression analysis of the 4 pre-selected peptides

Peptides	B	S.E.	Wald	df	Sig.	Exp(B)
1969.9	-0.009	0.005	3.09	1	0.079	0.991
2107.1	0.003	0.004	0.65	1	0.42	1.003
2213.4	-0.002	0.001	8.761	1	0.003	0.998
1947.9	0	0.001	0	1	0.999	1
Constant	6.993	2.367	8.73	1	0.003	1089

Table 6. Peptides used in the multivariate logistic regression analysis for developing the classification model (lung adenocarcinoma vs. healthy controls)

Peptides	B	S.E.	Wald	df	Sig.	Exp(B)	95.0% C.I. for EXP(B)	
							Lower	Upper
1969.9	-0.006	0.002	6.353	1	0.012	0.994	0.989	0.999
2213.4	-0.002	0.001	10.198	1	0.001	0.998	0.997	0.999
Constant	6.039	1.77	11.646	1	0.001	419.445		

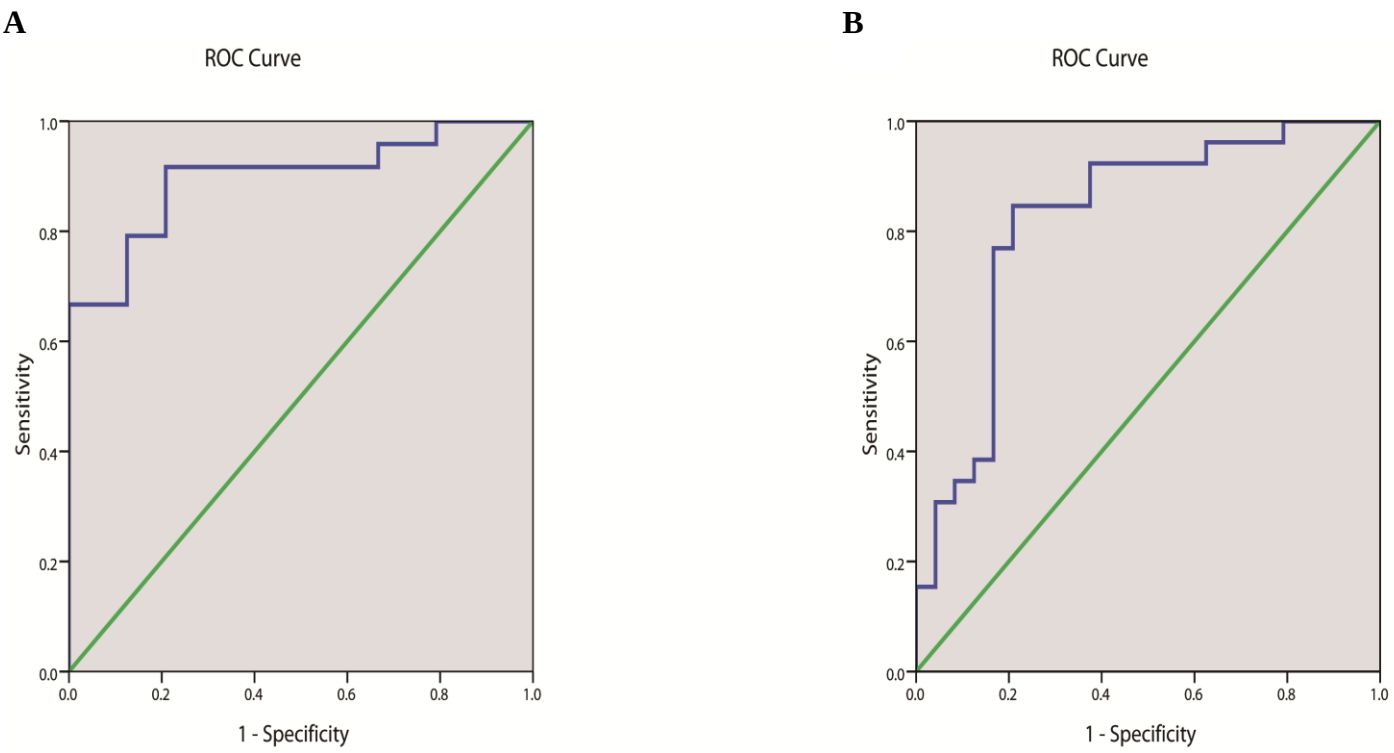


Figure1. ROC curve for (A) combination of peptides 1969.9 and 2213 Da, (B) combination of peptides 1781.8 and 1984.7 Da from multivariate logistic regression analysis

3.2 Modeling for lung adenocarcinoma patients using mass spectra data

For the purpose of discriminating lung adenocarcinoma patients from healthy controls, we constructed models using binary logistic regression analysis. The correlation of the 14 peptide peaks selected by ClinPro software was analyzed by spearman analysis. The peptides m/z 1969.9, 2107.1,2 213.4, 1947.9 Da which are not only a significant AUC for discrimination ($p<0.05$, table 4) but also relatively independent to each other ($r<0.7$, $p<0.01$) were substituted into the multivariate logistic regression, table 5. As shown in table 6, 1969.9 and 2213.4 Da enter the model for discriminating lung adenocarcinoma patients from healthy controls. The area under the curve of ROC curve (AUROC) is 0.898(Figure 1A) and the accuracy is 85.45%, with a sensitivity of 91.7% and a specificity of 79.2%. These data demonstrate the capability of mass spectra data to discriminate lung adenocarcinoma patients from healthy controls. The classification equation for discriminating lung adenocarcinoma patients from healthy controls is as follows:

$$p=[1+e^{(-0.006\times mass1969.9-0.002\times mass2213.4+6.039)}]^{-1}$$

3.2 Modeling for advanced lung adenocarcinoma with BM with mass spectra data

For the purposes of discriminating advanced lung adenocarcinoma with BM from patients without BM, we compare the difference of the above 14 peaks(table 3) in advanced lung adenocarcinoma patients with BM as compared with patients without BM, and only 5 peaks showed significant difference, table 7. As shown in table 8, peptides m/z 1781.8 and 1984.7 Da enters the model for discriminating advanced lung adenocarcinoma without BM from BM. The area under the curve of ROC curve (AUROC) is 0.822(Figure 1B) and accuracy is 82.0%, with a sensitivity of 84.6% and a specificity of 79.2%. The classification equation for discriminating

advanced lung adenocarcinoma patients with BM from patients without metastasis is as follows:

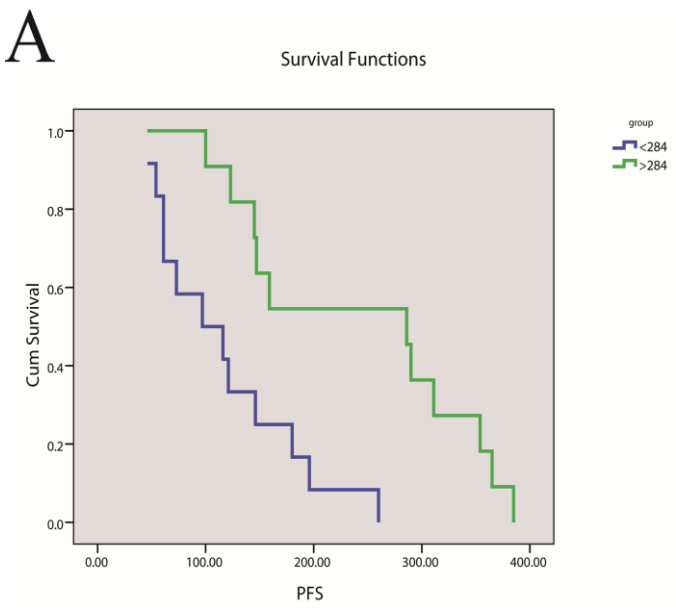
$$p=[1+e^{(-0.003\times mass1781.8+0.008\times mass1984.7+0.16)}]^{-1}$$

Table 7. Peptide peaks differentially expressed between lung adenocarcinoma patients without brain metastasis (NBM) and lung adenocarcinoma patients with brain metastasis(BM).

Peptides	NBM	BM	trend	p
1947.9	2033.4	2576.3	↑	0.004
4096.2	189.5	417.3	↑	0.006
1984.7	205.1	113.7	↑	0.008
1781.8	314.2	562.8	↑	0.042
2672	1246.3	687.1	↑	0.049

3.3 Prognostic Modeling for lung adenocarcinoma patients

The optimal cut-off value for the 14 serum peptides was determined by ROC analysis, Table 9. The patients were divided into 2 groups by these cut-off values. Prognostic values of the 14 serum peptides which were highly specific for lung adenocarcinoma were evaluated by univariate analysis, as indicated in Table 10.



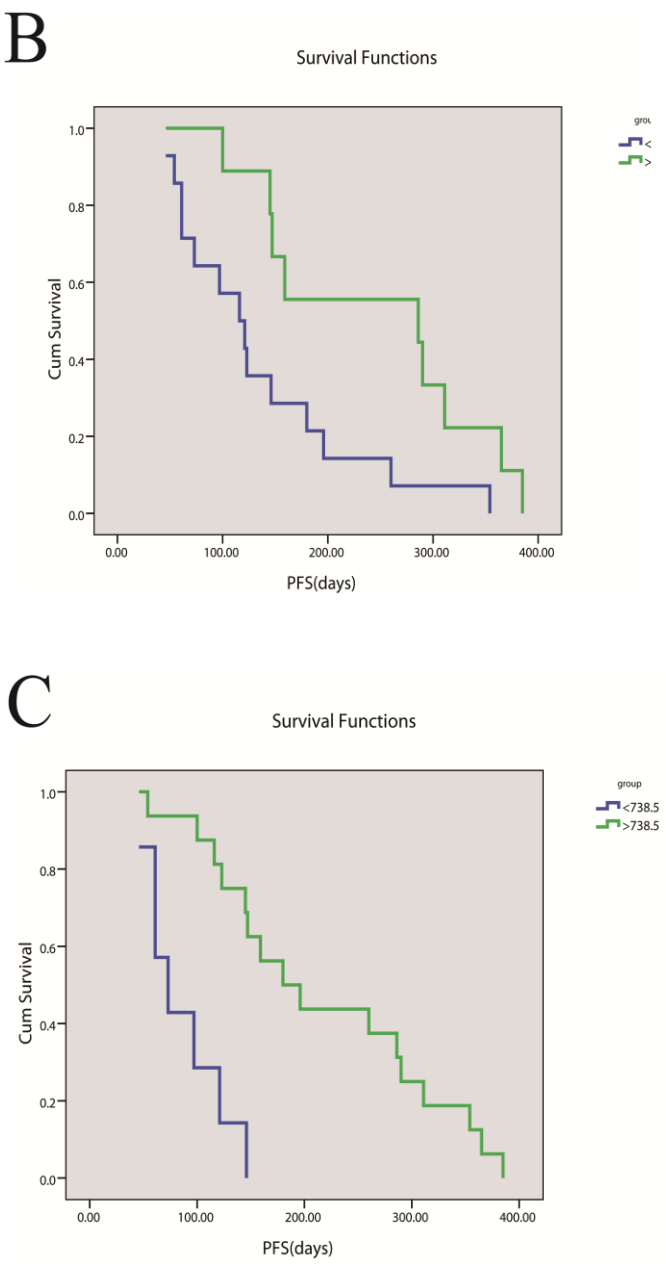


Figure 2. Kaplan–Meier survival curves of (A) peptiede m/z 1969.9 Da, (B) peptiede m/z 1984.7 Da, (C) peptiede m/z 2095.2 Da

The Kaplan-Meier analysis indicated that m/z 1969.9, 2095.2 and 1984.7 Da was significantly associated with the number of median PFS days, Table 10. A lower baseline 1969.9 (intensity≤284) was associated with an inferior outcomes in the univariate analysis when compared with patients with the intensity of m/z 1969.9>284 (median PFS, 97 versus 286 days, p=0.002, Table 10, Figure 2A). When stratified by peptide m/z 1984.7 Da (intensity >273 versus

intensity≤273), the difference in median PFS survival curves was statistically significant (116 versus 286 days, p=0.0019, Table 10, Figure 2B). Decreasing peptide m/z 2095.2 Da (≤154 versus >154) were also significantly associated with an unfavarable outcomes (median PFS,116 versus 286 days, p=0.0019, Table 10, Figure 2C). All the above parameters with a p-value< 0.05 were included in the multivariate Cox analysis. As summarized in Table 11, peptide m/z 1969.9 Da remained independent predictors of PFS (p=0.01) by multivariate analysis.

$$p=[1+e^{(-0.006\times mass1969.9)}]^{-1}$$

Table 8. Peptides used in the multivariate logistic regression analysis for developing the classification model (NBM vs. BM)

Peptides	B	S.E.	Wald	df	Sig.	Exp(B)	95.0% C.I.for EXP(B)	
							Lower	Upper
1781.8	-0.003	0.001	4.304	1	0.038	0.997	0.994	1
1984.7	0.008	0.005	2.79	1	0.095	1.008	0.999	1.017
Constant	0.16	0.77	0.043	1	0.835	1.174		

Table 9. List of the specificity, sensitivity and cutoff value for the 14 serum peptides which were evaluated to be specific for lung adenocarcinoma patien

Peptides	specificity	sensitivity	Cutoff values
1781.8	0.444	0.917	121.50
1869.1	0.333	0.792	313.00
1947.9	0.333	0.875	975.50
1969.9	0.889	0.542	284.00
1984.7	0.667	0.417	273.00
2095.2	1.000	0.292	154.00
2107.1	0.667	0.792	437.00
2213.4	0.778	0.875	2750.50
2663.8	0.889	0.542	619.50
2865.8	0.667	1.000	226.00
3244.4	0.778	0.750	738.50
4096.2	0.792	0.875	225.50
4214.1	0.333	0.875	975.50
5910.2	0.778	0.583	977.00

3.4 Prognostic Modeling for advanced lung adenocarcinoma patients BM

Pre-warning advanced lung adenocarcinoma patients with BM is important to improve quality of life for patients, we then investigated the prognostic value of peptides m/z 1969.9, 2095.2 and 1984.7 Da in predicting the prognosis of advanced lung adenocarcinoma patients with BM. As shown in table 12, peptide m/z 1984.7 Da shows favourable value on pre warning lung adenocarcinoma patients with BM.

$$p=[1+e^{(-0.002\times mass1984.7)}]^{-1}$$

4. Discussion

In present study, we have successfully detected a series of short peptides that differentially expressed in the serum of patients with lung adenocarcinoma as compared with healthy controls. We also constructed classification and prognostic models for lung adenocarcinoma patients with BM through MOLDI-TOF MS method.

Table 10. Univariate analysis of predictive values of the selected 14 peptides for lung adenocarcinoma patients.

Peptides	Group	Progression cases	Median PFS duration (days)			
			Estimate	Std Error	χ2	P
1781.8						
	low	7	180	97.163	0.066	0.797
	high	43	145	25.715		
1869.1						
	low	11	147	26.291	0.013	0.91
	high	39	145	26.517		
1947.9						
	low	9	54	7.5	0.989	0.32
	high	41	147	10.157		
1969.9						
	low	26	97	37.239	9.451	0.002
	high	24	286	78.719		
1984.7						
	low	30	116	22.45	5.536	0.019
	high	20	286	189.32		
2095.2						
	low	15	73	15.712	14.394	0
	high	35	180	37		
2107.1						
	low	39	123	26.517	0.448	0.503
	high	11	260	125.976		
2213.4						
	low	46	145	19.072	0.425	0.514
	high	4	147	.		
2663.8						
	low	24	116	14.313	0.695	0.404
	high	26	147	11.258		
2865.8						
	low	0				
	high	23				
3244.4						
	low	14	116	15.922	0.484	0.487
	high	36	147	9.604		
4096.2						
	low	6	61	12.247	0.142	0.707
	high	44	146	2.236		
4214.1						
	low	24	121	14.313	1.393	0.238
	high	26	147	11.258		26
5910.2						
	low	22	123	50.596	0.038	0.846
	high	28	146	15.578		

Table 11.Peptides used in the multivariate Cox regression analysis for developing the prognostic model for lung adenocarcinoma patients.

Steps	Peptides	B	SE	Wald	df	Sig.	Exp(B)	95.0% CI for Exp(B)	
								Upper	Lower
Step 1	1969.922	-0.01	0.006	2.616	1	0.106	0.99	1.002	0.979
	2095.167	0.003	0.002	1.686	1	0.194	1.003	1.008	0.998
	1984.683	0	0.003	0.015	1	0.902	1	1.006	0.993
Step 2	1969.922	-0.01	0.004	8.378	1	0.004	0.99	0.997	0.983
	2095.167	0.003	0.002	3.101	1	0.078	1.003	1.007	1
Step 3	1969.922	-0.006	0.002	6.623	1	0.01	0.994	0.999	0.989

Table 12. Peptides used in the multivariate Cox regression analysis for developing the prognostic model for lung adenocarcinoma patients with BM.

Steps	Peptids	B	SE	Wald	df	Sig.	Exp(B)	95.0% CI for Exp(B)	
								Upper	Lower
Step 1	1969.922	0.01	0.01	1.026	1	0.0311	1.01	1.029	0.991
	2095.167	-0.003	0.003	0.983	1	0.0321	0.997	1.003	0.992
	1984.683	-0.008	0.007	1.346	1	0.0246	0.992	1.006	0.978
Step 2	1969.922	0.001	0.004	0.097	1	0.0756	1.001	1.01	0.993
	1984.683	-0.003	0.005	0.439	1	0.0507	0.997	1.006	0.988
Step 3	1984.683	-0.002	0.002	0.655	1	0.0418	0.998	1.003	0.994

Current knowledge of cellular regulation indicates that many networks operate at the translational levels. Proteomic technologies will help further understand the interaction that connect the serum or plasma with tumor microenvironment. Peptidomic maps associated with lung adenocarcinoma were drawn in this study. In particular, the prominent peptides that have a greater than twofold change in intensity, such as m/z 1781.8,1869.1, 1969.9,1984.7, 2095.2, 2213.4, 2663.8, 3244.4, 4096. 2 and 4214.1 including 7 upregulated peptide (m/z 1781.8,1869.1, 1969.9, 2663.8, 3244.4, 4096. 2 and 4214.1,) and 3 downregulated peptides (m/z 1984.7, 2095.2 and 2213.4) were detected predominantly in serum from lung adenocarcinoma patients as compared to serum from healthy volunteers.

Of these, a cluster of two peaks at m/z 1969.9 and 2213.4 Da achieved a classification capacity with the accuracy of close to 85.45% (a specificity of 79.2%, and a sensitivity of 91.7%) to discriminate lung adenocarcinoma from healthy volunteers. The peak 1969.9 Da, which is a fragment of transthyretin precursor, was more highly expressed in lung adenocarcinoma patients than in healthy controls in our study. Previous data illustrated that peaks of transthyretin precursor (TTR) fragments have frequently been detected in papillary thyroid cancer, pancreatic carcinoma and meningioma after a MALLDI-TOF method (10,15,17). One of the peaks associated with TTR was reported to be overexpressed in papillary thyroid cancer and has been identified as fragment of TTR by 2-DE, MALDI-TOF/MS and Western blot (10). A fragment of TTR was reported as being

overexpressed in serum of pancreatic carcinoma patients receiving low dose of warfarin but not in those on high dose of warfarin using iTRAQ-coupled LC-MS/MS(17). Other studies have identified an specific cleavage fragment of TTR in human cerebrospinal fluid of patients with meningioma using two-dimensional electrophoresis and electrospray quadrupole time-of-flight tandem mass spectrometry analysis(15).All these suggested the potential of 1969.9 Da as a biomarker for cancer and the possible relationship between TTR and lung adenocarcinoma, which would be explored in our further work. In addition, peak m/z 2213.4 Da, another peptide in our model for lung adenocarcinoma patients, is one fragment of fibrinogen alpha chain precursor. Fragments of fibrinogen alpha chain precursor at m/z 1264.6Da and 3245.6Da have been identified as diagnostic biomarker for nasopharyngeal carcinoma (25), IgA nephropathy(11) and acute graft versus host disease (aGVHD)(27).

Model including peptides at m/z 1781.8 and 1984.683 Da achieved a accuracy of 82% with a sensitivity of 84.6% and a specificity of 79.2% to discriminate advanced lung adenocarcinoma with BM from patients without BM. Peptide m/z 1781.8 was identified as a degraded fragment of ADP-ribosylarginine hydrolase (ARH1),which can regulates cell proliferation and tumorigenesis(13). Meanwhile, Peptide 1984.7, a degraded derivative of thymosin beta-4-like protein 3(TMSL3), may be an significant prognostic biomarker for lung adenocarcinoma patients with BM. Gianazza et al also found an up-expressed fragment of TMSL3 at 5337.62 Da by MALDI-TOF MS serum in renal cell carcinoma(9).And the potential prognostic value of peptide 1984.7 remains to be further investigated

The m/z 1969.9 Da may also serve as an significant prognostic biomarker for lung adenocarcinoma patients while m/z 1984.7 Da serve as an significant prognostic biomarker for advanced lung adenocarcinoma with BM. Few paper have investigated the prognostic value of peptide 1969.9 and 1984.7 Da. Peptides m/z 1969.9 and 1984.7 Da may be defined as the leading differential peptides associated with prognosis, worthy of further sequence determination and function analysis.

Collectively, the classification and prognostic model we have set up will have application in providing information for diagnosis and prognosis of lung adenocarcinoma patients, and may provide a better understanding of brain metastasis in lung adenocarcinoma, finally resulting in an improvement in outcomes of patients. However, the sample size is limited and we will confirm the usefulness of our currently identified peptides in larger patient cohorts in further work. After this confirmation, we will then determine the function of the peptides of interest.

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