Identification of integrin β1 as a prognostic biomarker for human lung adenocarcinoma using 2D-LC-MS/MS combined with iTRAQ technology

PENG-FEI ZHANG[1], GU-QING ZENG[1,2], LUN-ZHAO YI[1,3], JIAN-PING LIU[1], XUN-XUN WAN[1], JIA-QUAN QU[1], JIAN-HUANG LI[1], CUI LI[1], CAN-E TANG[1], RONG HU[1], XU YE[1], YU CHEN[1], ZHU-CHU CHEN[1] and ZHI-QIANG XIAO[1]

[1]Key Laboratory of Cancer Proteomics of Chinese Ministry of Health, Xiangya Hospital, Central South University, Changsha, Hunan 410008; [2]School of Nursing, University of South China, Hengyang, Hunan 421001; [3]Research Center of Modernization of Chinese Herbal Medicine, College of Chemistry and Chemical Engineering, Central South University, Changsha, Hunan 410083, P.R. China

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Abstract. To discover novel lung adenocarcinoma (AdC) biomarkers, isobaric tags for relative and absolute quantitation (iTRAQ)-tagging combined with 2D-LC-MS/MS analysis was used to identify differentially expressed plasma membrane proteins in lung AdC and paired paraneoplastic normal lung tissues (PNLTs) adjacent to tumors. In this study, significant caveolin-1 downregulation and integrin β1 upregulation was observed in primary lung AdC vs. PNLT. As there has been no report on the association of integrin β1 with lung AdC, immunohistochemical staining was performed to detect the expression of integrin β1 in an independent set of archival tissue specimens including 42 cases of PLNT, 46 cases of without lymph node metastasis primary AdC (non-LNM AdC) and 62 cases of LNM AdC; the correlation of their expression levels with clinicopathological characteristics and clinical outcomes were evaluated. Based on the data, upregulation of integrin β1 was significantly correlated with advanced clinical stage and lymph node metastasis. Integrin β1 overexpression was significantly associated with advanced clinical stage ($P<0.05$), lymph node metastasis ($P<0.05$), increased relapse rate ($P<0.05$) and decreased overall survival ($P<0.05$) in AdCs. Cox regression analysis indicated that integrin β1 overexpression is an independent prognostic factor. The data suggest that integrin β1 is a potential biomarker for LNM and prognosis of AdC and integrin β1 upregulation may play an important role in the pathogenesis of AdC.

Correspondence to: Dr Peng-Fei Zhang or Professor Zhi-Qiang Xiao, Key Laboratory of Cancer Proteomics of Chinese Ministry of Health, Xiangya Hospital, Central South University, 87 Xiangya Road, Changsha, Hunan 410008, P. R. China
E-mail: zhangpf690421@csu.edu.cn
E-mail: zqxiao2001@hotmail.com

Abbreviations: AdC, lung adenocarcinoma; PM, plasma membrane; PNLT, paraneoplastic normal lung tissue; non-LNM AdC, without lymph node metastasis primary AdC; LNM AdC, with lymph node metastatic primary AdC; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SCX, strong cation exchange; ACN, acetonitrile; GRAVY, grand average of hydropathicity; iTRAQ, isobaric tags for relative and absolute quantitation

Key words: lung adenocarcinoma, tumor biomarker, iTRAQ, plasma membrane proteome

Introduction

Lung cancer remains the leading cause of cancer-related mortality in several countries, including China (1,2). Although significant improvements have been made in the diagnosis and treatment of lung cancer, the prognosis for lung cancer is poor, with an overall five-year survival rate of approximately 16% (3). Lung adenocarcinoma (AdC) is a common histological type of lung cancer. In recent years, the frequency of lung AdC has increased and its prognosis remains poor (4,5). This high mortality rate is often attributed to the presence of advanced-stage metastasis, with more than two thirds of patients showing lymph node involvement and metastasis at the initial diagnosis. Therefore, to develop effective new strategies for the prediction, diagnosis and treatment of lung cancer metastasis, molecular mechanisms controlling metastasis must be identified.

Several groups have successfully used gene expression profiling techniques and model systems with different invasive or metastatic ability to identify genes that correlate with invasiveness or metastatic potential (6-8). Proteomics, particularly quantitative proteomics, has introduced a new approach to cancer research which aims at identifying differential expression proteins associated with carcinogenesis, providing new opportunities to reveal the molecular mechanism underlying this disease. Identification of differentially expressed proteins in lung AdC using proteomics revealed that expression levels of proteins may have some predictive power for metastasis and prognosis (9-12). In our lab, comparative proteomic studies of

primary lung AdC with and without lymph node metastasis suggest that Annexin A3 and Annexin A1 are potential biomarkers for lymph node metastasis and prognosis in lung AdC (13,14).

However, in the past years, lung AdC proteomic studies have focused on whole cellular proteomic analysis. Subcellular proteomic analysis has advantages over whole cellular proteomic analysis, including its ability to identify low-abundance proteins that may play a crucial role in tumors and provides a deeper insight into cellular events as protein abundances can be revealed on the level of different subcellular compartments and also protein translocations between different cell parts can be detected (15,16). The cell membrane possesses a number of important biological functions, such as signaling transduction into and out of the cells, ion transport and cell-cell and cell-matrix interactions and communications (17,18). Plasma membrane (PM) proteins are known to have implications in cell proliferation, cell adhesion, cell motility and tumor cell invasion (19-21) and account for more than two thirds of currently known drug targets (22,23). Therefore, the cell membrane is of substantial interest with regard to various aspects of tumor, from carcinogenic and metastatic mechanisms to molecular diagnosis and therapeutics. A membrane proteomic analysis offers unprecedented possibilities for identification of tumor biomarkers and therapeutic targets and for understanding carcinogenic mechanisms.

In the present study, isobaric tags for relative and absolute quantitation (iTRAQ) labeling followed by 2D-LC-MS/MS was performed to identify differential PM proteins in AdC tissues and paired normal lung tissues adjacent to tumors. Two different proteins, caveolin-1 and integrin β1, identified by the quantitative proteomics, were selected for validation by western blotting. Furthermore, the clinicopathological significance of integrin β1 was further evaluated using immunohistochemistry of paraffin-embedded archival tissue specimens and statistical analyses. Our data facilitate an understanding of AdC carcinogenesis and mining biomarkers for the diagnosis and treatment of this disease.

Materials and methods

Tissue specimens. Twenty cases of fresh primary lung AdCs and paired paraneoplastic normal lung tissues (PNLTs) adjacent to tumors from the lung AdC patients undergoing curative surgery were obtained from the Department of Cardiothoracic Surgery, Xiangya Hospital of Central South University, China, and stored at -80˚C until use. The patients signed an informed consent form for the study which was approved by the local Ethics Committee. Two pairs of matched tumor and normal tissues were used for iTRAQ labeling and eighteen pairs of matched tumor and normal tissues were used for western blotting. An independent set of formalin-fixed and paraffin embedded archival tissue specimens used for immunohistochemistry were obtained from the Department of Pathology, Xiangya Hospital of Central South University and included 42 cases of PNLT, 46 cases of without lymph node metastasis primary AdC (non-LNM AdC) and 62 cases of with lymph node metastatic (LNM) AdC between January 2004 and May 2006 from the AdC patients undergoing curative surgery. The patients recruited in this study had not received chemotherapy, radiotherapy prior to the surgery. The clinicopathological characteristics of the patients used in the present study are noted in Table I.

Purification of PM. PM was purified using sucrose density centrifugation in combination with aqueous two-phase partition as described by Cao *et al* (24). Ten samples were pooled to purified PM for each AdC and PNLT. The purified PM fractions were pelleted by centrifugation and frozen at -80˚C until used for protein extraction.

Protein extraction, digestion and labeling with iTRAQ reagents. The PMs were dissolved in lysis buffer (7 M urea, 2 M thiourea, 65 mM DTT, 0.1 mM PMSF) at 4˚C for 1 h and then centrifuged at 12,000 rpm for 30 min at 4˚C. The supernatant was collected and desalted using 2D Cleanup Kit (Amersham Biosciences). The protein concentration was determined by 2D Quantification Kit (Amersham Biosciences). Trypsin digestion and iTRAQ labeling were performed according to the manufacturer's protocol (Applied Biosystems). Briefly, 100 μg protein sample was reduced and alkylated and then digested overnight at 37˚C with 1 mg/ml trypsin solution and labeled with iTRAQ™ Reagents (Applied Biosystems). The iTRAQ labeling was labeled with 114 and 116 iTRAQ tags for lung AdC samples and 115 and 117 iTRAQs for normal lung tissue samples. Four labeled digests were then mixed and dried using a rotary vacuum concentrator.

LC-MS/MS. The mixed peptides were fractionated by strong cation exchange (SCX) chromatography on an LC-20AD HPLC system (Shimadzu) using a polySulfoethyl column (2.1x100 mm, 5 μm, 300 Å; The Nest Group, Inc.) as previously described by us (25). Briefly, the mixed peptides were desalted with Sep-Pak Cartridge (Waters), diluted with the loading buffer [10 mM KH_2PO_4 in 25% acetonitrile (ACN), pH 2.8] and loaded onto the column. Buffer A was identical in composition to the loading buffer and buffer B was the same as buffer A except that it contained 350 mM KCl. Separation was performed using a linear binary gradient of 0-80% buffer B in buffer A at a flow rate of 200 μl/min for 60 min. The absorbance at 214 and 280 nm was monitored and a total of 30 SCX fractions were collected along the gradient.

Each SCX fraction was dried down by the rotary vacuum concentrator, dissolved in buffer C (5% ACN, 0.1% FA) and analyzed on Qstar XL (Applied Biosystems) as previously described by us (25). Briefly, peptides were separated on a reverse-phase (RB) column (ZORBAX 300SB-C18 column, 5 μm, 300 Å, 0.1x15 mm; Micromass) using an LC-20AD HPLC system. The HPLC gradient was 5-35% buffer D (95% ACN, 0.1% FA) in buffer C at a flow rate of 0.2 μl/min for 65 min. Survey scans were acquired from 400-1,800 with up to 4 precursors selected for MS/MS from m/z 100-2,000 using a dynamic exclusion of 30 S. The iTRAQ labeled peptides fragmented under CID conditions to give reporter ions at 114.1, 115.1, 116.1 and 117.1 Th. The ratios of peak areas of the iTRAQ reporter ions reflect the relative abundances of the peptides and, consequently, the proteins in the samples. Larger sequence-information-rich fragment ions were also produced under these MS/MS conditions and gave the identity of the protein from which the peptide originated.

Table I. Relationship between integrin β1 expression and clinicopathological factors in lung adenocarcinoma.

		Integrin β1			
	N	Low (0-2)	Moderate (3-4)	High (5-6)	P-value
Age					0.87
<50	20	7	6	7	
≥50	88	31	29	28	
Gender					0.908
Male	60	25	20	15	
Female	48	13	15	20	
Differentiation					0.893
Well	22	8	7	7	
Moderate	23	8	8	7	
Poor	63	22	20	21	
pT stage					0.072
T1	22	11	10	1	
T2	63	29	33	1	
T3-4	23	4	6	13	
pN stage					0.024[a]
pN0	34	27	6	1	
pN1	32	9	21	2	
pN2	42	1	8	33	
Clinical stage					0.016[a]
I	42	30	11	1	
II	24	6	18	0	
III-IV	42	2	6	34	
Recurrence					0.000[a]
Negative	26	16	7	3	
Positive	82	12	28	32	

[a]P<0.05 by Mann-Whitney U test.

Data processing. The software used for data acquisition was Analyst QS 1.1 (Applied Biosystems). The software used for protein identification and quantitation was ProteinPilot™ 3.0 software (Applied Biosystems). The software compares relative intensity of proteins present in samples based on the intensity of reporter ions released from each labeled peptide and automatically calculates protein ratios and P-values for each protein. The data from LC-MS/MS analyses were merged and searched against combined human Swiss-Prot protein sequence database. The following search parameters were used: iTRAQ 4-plex as the sample type, digestion with trypsin and cystein alkylation with methyl methane thiosulfate. The precursor tolerance was set to 150 ppm and the iTRAQ fragment tolerance was set to 0.2 Da, one of missed cleavages permitted, fixed and variable modifications as well as the peak list generating parameters are built-in functions of ProteinPilot. Identified proteins were grouped by the software to minimize redundancy. All peptides used for the calculation of protein ratios were unique to the given protein or proteins within the group and peptides that were common to other isoforms or proteins of the same family were ignored. The protein confidence threshold cutoff was set to 1.3 (unused) with at least one peptide above the 95% confidence level. The average iTRAQ ratios from the two experiments were calculated for each protein. In addition, false discovery rate for the protein identification was calculated by searching against a reversed database.

Bioinformatics analysis. Predictions for putative transmembrane domains (TMDs) in all identified proteins were carried out using the transmembrane hidden Markov model (TMHMM) algorithm available at http://www.cbs.dtu.dk/services/TMHMM (26). The average hydropathy for identified proteins and peptides was calculated using the ProtParam software available at http://www.expasy.org (27). Proteins with positive grand average of hydropathicity (GRAVY) values were considered to be hydrophobic and those with negative values, hydrophilic.

Differential protein validation. Eighteen pairs of matched lung AdC and normal lung tissues were used for western blotting. Briefly, 50 μg of lysates were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a PVDF membrane. Blots were blocked with 5% nonfat dry milk for 2 h at room temperature and then incubated with primary anti-caveolin-1, or anti-integrin β1 antibody overnight at 4˚C, followed by incubation with a horseradish peroxidase-conjugated secondary antibody (1:3,000; Amersham Biosciences) for 1 h at room temperature. The signal was visualized with an ECL detection reagent and quantities by densitometry using Image J software (http://rsb.info.nih.gov/ij). β-actin was detected simultaneously as a loading control.

Immunohistochemical analysis. Immunohistochemistry was performed on formalin-fixed and paraffin-embedded tissue sections using a standard immunohistochemical technique. Briefly, 4 μm of tissue sections were deparaffinized, rehydrated and treated with an antigen retrieval solution (10 mmol/l sodium citrate buffer, pH 6.0). The sections were incubated with anti-integrin β1 antibody (1:40) overnight at 4˚C and were then incubated with 1:1,000 dilution of biotinylated secondary antibody followed by avidin-biotin peroxidase complex (DAKO) according to the manufacturer's instructions. Finally, tissue sections were incubated with 3',3'-diaminobenzidine (Sigma-Aldrich) until a brown color developed and counterstained with Harris modified hematoxylin. In negative controls, primary antibodies were omitted.

Immunostaining was blindly evaluated by two investigators in an effort to provide a consensus on staining patterns by light microscopy. A quantitative score was performed by adding the score of staining area and the score of staining intensity for each case to assess the expression levels of the proteins as previously described by us (28). A combined staining score of ≤2 was considered to be low staining (no expression), a score between 3 and 4 was considered to be moderate staining (expression), and a score between 5 and 6 was considered to be strong staining (high expression).

Table II. Forty-five differentially expressed proteins in AdC vs. matched PNLT identified by iTRAQ labeling combined with 2D-LC-MS/MS.

Accession no.	Protein name	AdC vs. PNLT	Located	TMDs	GRAVY
Q9UGM3	Deleted in malignant brain tumor 1 protein	40.005	Secreted	0	-0.346
P06731	Carcinoembryonic antigen-related cell adhesion molecule 5	13.094	PM	0	-0.411
P15941	Mucin-1	9.713	PM	1	-0.501
P05362	Intercellular adhesion molecule 1	9.034	PM	1	-0.326
P01833	Polymeric immunoglobulin receptor	6.916	PM	1	-0.405
Q99828	Calcium and integrin-binding protein 1	5.191	PM	0	-0.329
Q14764	Major vault protein	3.965	Cytoplasm	0	-0.361
P04406	Glyceraldehyde-3-phosphate dehydrogenase	3.837	Cytoplasm	1	-0.114
O96009	Napsin-A	3.768	Secreted	0	0.193
P08575	Leukocyte common antigen	3.375	PM	2	-0.628
A7Y9J9	Mucin-5AC	3.150	Secreted	0	-0.379
P98088	Mucin-5AC (fragments)	2.959	Secreted	0	-0.379
O00299	Chloride intracellular channel protein 1	2.619	PM	0	-0.293
Q00610	Clathrin heavy chain 1	2.606	M	0	-0.244
P08311	Cathepsin G	2.503	M	0	-0.562
P05023	Sodium/potassium-transporting ATPase subunit α-1	2.456	PM	10	0.010
P04083	Annexin A1	2.453	M Related	0	-0.426
O75955	Flotillin-1	2.281	PM	0	-0.338
P01871	Ig mu chain C region	2.240	PM	0	-0.326
P06733	α-enolase	1.720	M	0	-0.226
P05556	Integrin β1	1.583	PM	1	-0.455
P27105	Erythrocyte band 7 integral membrane protein	0.496	PM	1	0.037
Q99536	Synaptic vesicle membrane protein VAT-1 homolog	0.490	Cytoplasm	0	-0.043
Q9BVC6	Transmembrane protein 109	0.442	PM	5	0.411
P11233	Ras-related protein Rap-1A	0.436	PM	0	-0.683
Q969L2	Protein MAL2	0.436	PM	4	0.728
Q99758	ATP-binding cassette sub-family A member 3	0.393	PM	11	0.089
P07099	Epoxide hydrolase 1	0.387	PM	0	-0.261
P21333	Filamin-A	0.341	PM	0	-0.318
P62158	Calmodulin	0.285	PM	0	-0.671
O60437	Periplakin	0.284	M	0	-0.982
P21964	Catechol O-methyl-transferase	0.236	PM	1	0.16
Q9UGT4	Sushi domain-containing protein 2	0.229	PM	1	-0.275
P00387	NADH-cytochrome b5 reductase 3	0.223	PM	0	-0.187
P08758	Annexin A5	0.223	M related	0	-0.337
P02730	Band 3 anion transport protein	0.160	PM	11	0.213
O00159	Myosin-Ic	0.148	PM	0	-0.387
Q16853	Membrane primary amine oxidase	0.147	PM	1	-0.135
P50148	Guanine nucleotide-binding protein G(q) subunit α	0.123	PM	0	-0.440
Q03135	Caveolin-1	0.115	PM	1	0.054
P10301	Ras-related protein R-Ras	0.086	PM	0	-0.408
Q9NZN4	EH domain-containing protein 2	0.057	PM	0	-0.309
Q09666	Neuroblast differentiation-associated protein AHNAK	0.057	Nucleus	0	-0.499
Q6NZI2	Polymerase I and transcript release factor	0.053	M	0	-0.272
P22748	Carbonic anhydrase 4	0.024	PM	1	-0.576

AdC, lung adenocarcinoma; PM, plasma membrane; PNLT, paraneoplastic normal lung tissue; TMDs, transmembrane domains; GRAVY, grand average of hydropathicity.

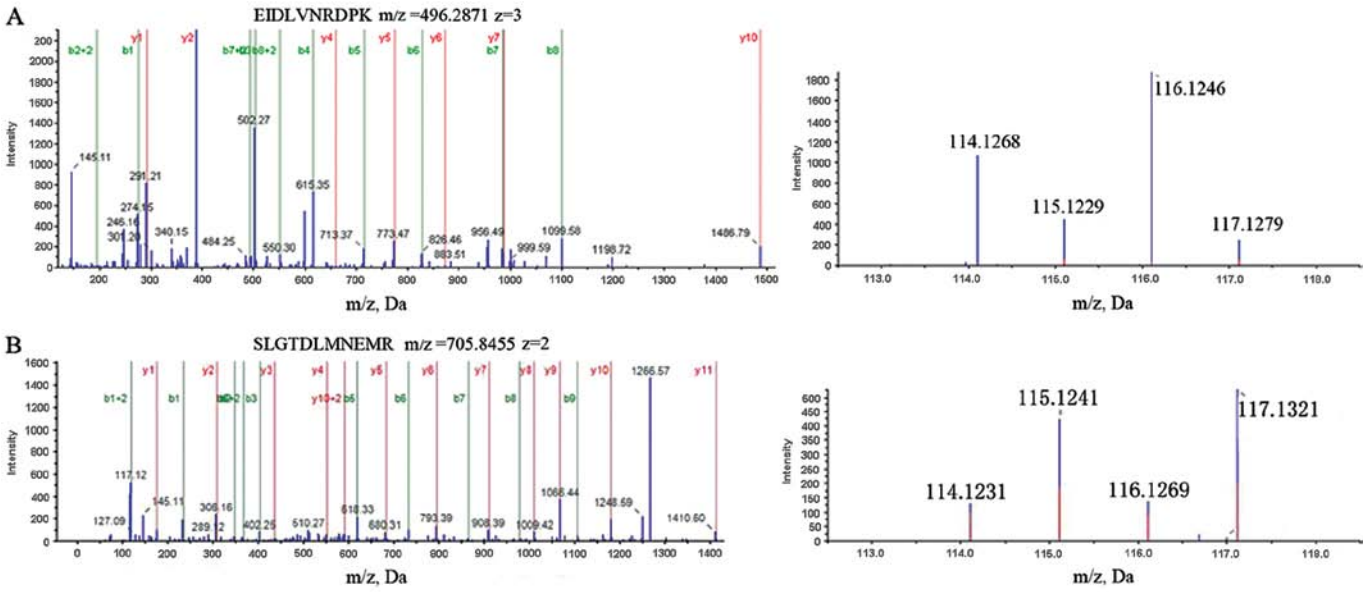

Figure 1. MS/MS spectra used for the identification and quantitation of caveolin-1 and integrin β1 with labeling 115 and 117 tags for AdC samples, and 114 and 116 tags for normal lung tissue samples. (A) Left, the MS/MS spectra of precursor ion EIDLVNRDK with m/z of 496.2871 (z=3) for identified caveolin-1; right, relative quantitative of caveolin-1 according to the signal intensity ratio for reporters 115:114 and 117:116. (B) Left, the MS/MS spectra of precursor ion SLGTDLMNEMR with m/z of 705.8455 (z=2) for identified integrin β1; right, relative quantitative of integrin β1 according to the signal intensity ratio for reporters 115:114 and 117:116.

Statistical analysis. All statistical analyses were performed using SPSS 13.0 Software. The significant difference integrin β1 expression between the tumor and normal tissues and primary and metastatic tumors was determined by using the Mann-Whitney U test. Significant differences between the expression of those two factors and clinical variables, including age, gender, histologic type/grade, primary tumor (T) stage, regional lymph node (N) metastasis, clinical stage and recurrence, were compared by the Mann-Whitney U test or ANOVA test. All patients underwent postoperative chemotherapy and were followed-up by telephone to obtain the information of patient outcome. The follow-up period lasted up to 60 months. Relapse-free duration was calculated from the time of surgery to the time of first recurrence after surgery. Overall survival was calculated from the time of surgery to the time of death. Mortality due to lung AdC was considered as outcome; mortality due to other causes was censored and the missing values were replaced by the series mean method. Relapse-free probability curves and overall survival curves were obtained by the Kaplan-Meier method and log-rank testing was used to evaluate the statistical significance of differences. Cox regression analysis was used to evaluate the prognostic significance of clinicopathological factors. A difference of $P<0.05$ was considered statistically significant.

Results

Identification of differentially expressed proteins in lung AdC and normal lung tissue using iTRAQ-2D-LC-MS/MS. The 2D-LC-MS/MS analysis resulted in the identification of 353 proteins (data not shown) in iTRAQ labeling experiment, with ≥95% confidence, using one or more peptides. We also analyzed identified proteins on the basis of subcellular location and predicted TMDs. Among the proteins with their location annotated, proteins located on PM are known as PM proteins. Of the 353 identified proteins by MS/MS analysis, 224 (63.5%) proteins are PM or PM-related proteins. Of these, 91 (25.8%) are predicted to have one or more TMDs. The GRAVY values of identified PM proteins range from -1.165 to 1.027.

In the present study, the proteins that met the following criteria were considered as differential proteins between the two types of tissues: i) proteins were identified based on ≥2 peptides in the iTRAQ labeling experiments; ii) proteins were quantified with at least two peptides; iii) P-value of identified proteins <0.05; and iv) proteins showed an averaged ratio-fold-change ≥1.5 or ≤0.66. According to these criteria, a total of 45 differentially expressed proteins were found in the two types of tissues, 21 proteins upregulated and 24 downregulated (Table II). MS/MS spectra used for the identification and quantitation of caveolin-1 and integrin β1 are shown in Fig. 1.

Validation of differentially expressed proteins identified by quantitative proteomics. To confirm the expression levels of the differential proteins identified by a proteomics approach, expressions of caveolin-1 and integrin β1 in 18 pairs of AdC and matched PNLT adjacent to tumors were detected by western blotting. As shown in Fig. 2, caveolin-1 was downregulated, whereas integrin β1 was upregulated in AdC compared with PNLT, which is consistent with the findings in MS/MS analysis.

Expression of integrin β1 in PNLT, primary lung AdC and lymph node metastases. We detected the expression of integrin β1 using immunohistochemical staining in 46 cases of non-LNM AdC, 62 cases of LNM AdC and 42 cases of PNLT. As shown in Fig. 3 and Table III, integrin β1 was significantly upregulated in non-LNM AdC vs. PNLT and in LNM AdC vs. non-LNM AdC.

Table III. Difference of integrin β1 expression in PNLT, non-LNM AdC and LNM AdC.

	n	Score			P-value
		Low (0-2)	Moderate (3-4)	High (5-6)	
Integrin β1					
PNLT	42	34	6	2	0.001[a,b]
Non-LNM AdC	46	21	20	5	
LNM AdC	62	17	15	30	0.001[a,c]

[a]P<0.05 by Mann-Whitney U test. [b]PNLT vs. non-LNM AdC; [c]non-LNM AdC vs. LNM AdC. PNLT, paraneoplastic normal lung tissue; non-LNM AdC, lung AdC without lymph node metastasis; LNM AdC, lung AdC with lymph node metastasis.

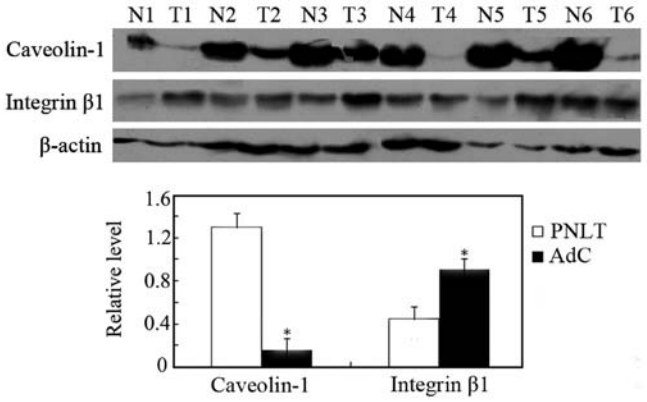

Figure 2. Representative results of western blotting of caveolin-1 and integrin β1 in the tissue samples and histogram of relative changes in the expression levels of integrin β1 in tumor and normal tissues as determined by densitometric analysis. β1-actin was used as a loading control.

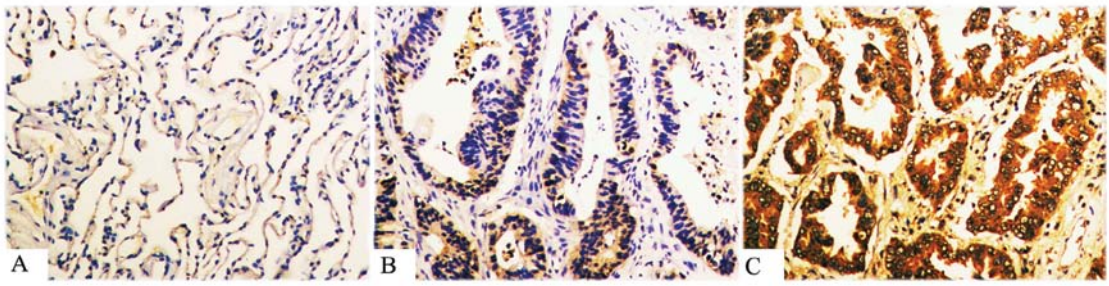

Figure 3. Representative results of immunohistochemistry of integrin β1 in tissue specimens. (A) Weak staining of integrin β1 in normal lung tissue; (B) moderate staining of integrin β1 in primary AdC tissue; (C) strong staining of integrin β1 in lymph node metastasis AdC tissue. Integrin β1 was expressed in membrane and cytoplasm. Presence of the specific protein is indicated by the amount of brown staining. Nuclei were counterstained with hematoxylin (blue) for visualization purposes. Original magnification, x200.

Correlation of integrin β1 expression in primary AdC with clinicopathological factors. Table I shows the correlation of several clinical pathological factors with integrin β1 expression status in 108 cases of primary AdC (including 46 cases of non-LNM AdC and 62 cases of LNM AdC). Integrin β1 expression levels were significantly correlated with clinical stage, recurrence and lymph node metastasis. Tumors with the upregulation of integrin β1 tended to have a more advanced clinical stage and more frequent recurrence and lymph node metastasis. No significant correlations were found between the expression of integrin β1 and other characteristics, including gender, age, tumor size and tumor differentiation.

Correlation of postoperative relapse and survival with integrin β1 expression. By the end of the study, 82 of the 108 patients had died, 24 patients were still alive and 2 patients had been lost to follow-up. The relapse-free times of the patients with low, moderate and high expression of integrin β1 were 51.6±10.1, 47.1±13.3 and 16.5±10 months, respectively. The relapse-free probability curve showed that the relapse rate was significantly increased along with increasing integrin β1 expression (Fig. 4, left). The mean survival rates of the patients with low, moderate and high expression of integrin β1 were 53±8.7, 38.4±12.9 and 17.8±9.5 months, respectively. The survival curves showed that the overall survival rate was significantly decreased along with increasing expressions of integrin β1 (Fig. 4, right). In univariate analysis (Table IV), increased postoperative relapse and decreased survival were correlated with advanced clinical stage, lymph node metastasis, increasing expressions of integrin β1. In multivariate analysis (Table V), advanced clinical stage, lymph node metastasis and increasing expressions of integrin β1 remained the significant independent prognostic factors of increased relapse rate and decreased overall survival rate.

Discussion

In the present study, iTRAQ labeling combined with 2D-LC-MS/MS was performed to identify differential PM proteins in AdC and PNLT. As a result, 45 differentially expressed proteins were identified and differential PM proteins, caveolin-1 and integrin β1, were selectively validated. Next, the clinicopathological significance of integrin β1 was further evaluated using immunohistochemistry of paraffin-embedded archival tissue specimens and statistical analysis. Results show that integrin β1 is a potential biomarker for LNM and prognosis of AdC.

Integrins, a large family of membrane receptors, are α/β-heterodimeric transmembrane adhesion molecules which bind to specific ECM ligands (29,30). Integrin β1 is an impor-

Table IV. Univariate Cox regression analysis of relapse-free and overall survival for integrin β1 expression.

	Relapse-free probability		Overall survival	
Variables	HR (95% CI)	P-value	HR (95% CI)	P-value
Age	0.501(0.308-0.813)	0.094	0.465 (0.287-0.753)	0.120
Gender	0.926(0.596-1.438)	0.731	0.929 (0.596-1.452	0.747
Male (reference)	1.000		1.000	
Female	0.926(0.596-1.438)	0.731	0.929 (0.595-1.452)	0.747
Tumor differentiation		0.700		0.502
Well (reference)	1.000		1.000	
Moderate	0.871 (0.486-1.56)	0.426	1.384 (0.681-2.811)	0.369
Poor	1.504 (0.618-1.796)	0.750	1.210 (0.769-1.904)	0.410
pT stage		0.513		0.779
T1 (reference)	1.000		1.000	
T2	0.675 (0.341-1.336)	0.611	1.009 (0.352-2.895)	0.861
T3-4	0.781 (0.449-1.360)	0.257	1.288 (0.560-2.962)	0.551
pN stage		0.000[a]		0.000[a]
N0 (reference)	1.000		1.000	
N1	14.812 (6.024-36.421)	0.000	16.639 (6.513-42.507)	0.000
N2-3	11.357 (6.244-20.660)	0.000	11.988 (6.518-22.045)	0.006
Clinical stage		0.000[a]		0.000[a]
I (reference)	1.000		1.000	
II	19.744 (7.291-53.464)	0.000	18.973 (7.073-50.899)	0.000
III-IV	21.445 (10.799-42.585)	0.000	20.501 (13.340-44648)	0.000
Integrin β1 expression		0.000[a]		0.000[a]
Low	1.000		1.000	
Moderate	2.202 (1.231-3.935)	0.008	2.626 (1.442-4.780)	0.002
High	6.592 (4.106-10.584)	0.000	6.183 (3.851-9.929)	0.000

[a]Overall P<0.05. HR, hazard ratio; CI, confidence interval.

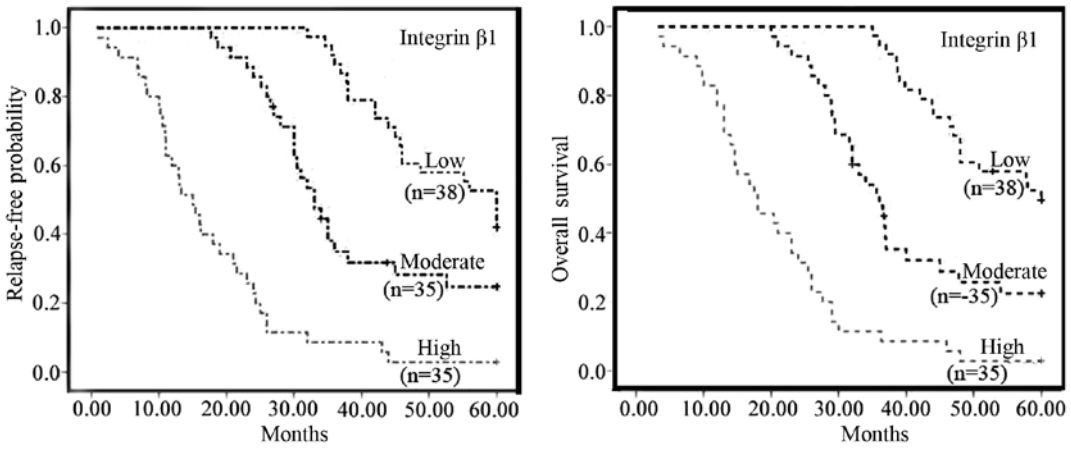

Figure 4. Kaplan-Meier survival plots for lung adenocarcinoma patients according to the expression levels of integrin β1. (Left) Integrin β1 expression and probability of being relapse-free. (Right) Integrin β1 expression and overall survival. P-value was determined using a two-sided log-rank test.

tant subunit of integrins and recognizes the sequence R-G-D in a wide array of ligands. Integrin β1 comprises at least eight isoform members and plays a role in cell signaling and thereby defines cellular shape, mobility and regulates the cell cycle (31). Integrin β1 has been shown to regulate signaling through transmembrane growth factor receptors such as

Table V. Multivariate Cox regression analysis of relapse-free and overall survival for integrin β1 expression.

	Relapse-free probability		Overall survival	
Variables	HR (95% CI)	P-value	HR (95% CI)	P-value
pN stage		0.011[a]		0.006[a]
N0 (reference)	1.000		1.000	
N1	5.354(1.537-18.644)	0.008	7.484(2.040-27.463)	0.002
N2-3	3.508(1.493-8.240)	0.004	3.551(1.477-8.537)	0.005
Clinical stage		0.003[a]		0.005[a]
I (reference)	1.000		1.000	
II	4.440(1.186-16.619)	0.027	4.165(1.130-15.348)	0.032
III-IV	5.167(2.028-13.163)	0.001	4.745(1.862-12.094)	0.001
Integrin β1 expression		0.000[a]		0.000[a]
Low	1.000		1.000	
Moderate	2.086(1.010-4.310)	0.047	2.866(1.311-6.265)	0.008
High	4.045(2.169-7.543)	0.000	3.557(1.879-6.734)	0.000

[a]Overall P<0.05. HR, hazard ratio; CI, confidence interval.

epidermal growth factor receptor and transforming growth factor-β receptor (32,33). Integrin β1 has been reported as critical for TGF-β1-mediated transcription and epithelial cell plasticity *in vitro* (34). Integrin β1 was involved in the development and progression of carcinogenesis in several types of cancer, including kidney cancer, breast cancer and fibrosarcoma, bladder and colon carcinoma (34,35). Bredin *et al* (36) found that integrin β1 was involved in lung cancer cell migration *in vitro* towards fibronectin, laminin and type IV collagen. Upregulation of integrin β1 is an important factor for gefitinib resistance in the NSCLC cell line (37) and overexpression of integrin β1 is correlated with the invasion and metastasis events of HCC in patients (38). Integrin β1 is correlated with highly invasive and metastatic behavior and is a poor prognostic factor in patients with SCLC (39,40). Herein, an increase in integrin β1 expression level was associated with advanced clinical stage and lymph node metastases, suggesting that integrin β1 is associated with the progression and LNM of lung AdC. In addition, a univariate analysis indicated that high integrin β1 expression is strongly associated with increased tumor relapse and a multivariate analysis further indicated that high integrin β1 expression is an independent relapse factor for increased tumor relapse in lung AdC.

In conclusion, the present study not only confirmed expression of caveolin-1 and integrin β1 by proteomic approaches in primary AdC and paired normal lung tissues adjacent to tumors, but also showed that primary AdC with higher integrin β1 expression tended to have later clinical stage, more frequent recurrence and LNM. Furthermore, survival curves showed that the AdC patients with integrin β1 upregulation had a poor prognosis. Multivariate analysis confirmed that integrin β1 expression was an independent prognostic indicator. Findings of the present study may have clinical value in predicting the prognosis of AdC and identifying AdC patients that are at high risk of metastasis and recurrence.

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