Journal of Chromatography B, 878 (2010) 3395–3401

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Comparative analysis of the human urinary proteome by 1D SDS-PAGE and chip-HPLC-MS/MS identification of the AACT putative urinary biomarker

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ARTICLE INFO

Article history:
Received 12 August 2010
Accepted 25 October 2010
Available online 4 November 2010

Keywords:
Biomarker
Lung cancer
One-dimensional gel electrophoresis
Proteome
Urine

ABSTRACT

Urine is one of the most attractive analyte used for clinical diagnosis. NSCLC (non-small cell lung carcinoma), which includes adenocarcinoma, squamous cell carcinoma and large-cell carcinoma, is a leading cause of cancer-related deaths. In the present study, urinary proteomes of normal individuals and NSCLC patients were compared using 1D SDS-PAGE. From the distinctly differentially expressed bands in SDS-PAGE gel, 40 proteins were identified by chip-HPLC-MS/MS, including five proteins relevant to NSCLC. One of the selected proteins, alpha-1-antichymotrypsin (AACT), was further validated in urine by western blot and in lung tissue by immunohistochemistry staining. Higher expression level of AACT in NSCLC patients was observed by western blot when compared with normal urine samples. Significantly, the NSCLC tumor tissue (18 out of 20 cases, 90%) showed a significantly higher expression level of AACT compared to adjacent non-tumor lung tissue (3 out of 20 cases, 15%). These results establish AACT as a potential biomarker for objective and non-invasive diagnosis of NSCLC in urine and the other four NSCLC-related proteins were also listed.

1. Introduction

Urine is produced by kidney and eliminates waste products from the blood. It is well known that urine can be obtained non-invasively, in large quantities and that it is stable compared to other biofluids, it has become one of the most attractive biosample in clinical diagnosis [1]. The advent of soft ionization methods such as electrospray (ESI) and matrix assisted laser ionization/desorption (MALDI) MS-based proteomics has enabled a new approach to disease related biomarker discovery in urine. Although, mainly because urine was produced in the kidney and urinary tract, the majority of the proteomic researches of urine were applied to the kidney and urinary tract related disease. But recent reports show that the analysis of the urinary proteome can also be highly informative on the non-urogenital diseases and can also be used in their classification [2–4]. Of the total urinary proteome of healthy individuals, 70% originates from the kidney and urinary tract while the remaining 30% represents proteins filtered by the glomerulus. Therefore, the urine may contain information, not only on the urinary tract and the kidney, but also from other distant organs such as the liver and lung via blood, obtained by glomerular filtration [5]. The analysis of the urinary proteome might therefore allow the identification of biomarkers of both urogenital and systemic diseases such as liver cancer.

Lung cancer has a 5-year survival rate of approximately 15% and is a leading cause of cancer-related deaths. Non-small cell lung carcinoma (NSCLC), which includes adenocarcinoma, squamous cell carcinoma and large-cell carcinoma, accounts for 80% of the lung cancer cases [6]. Detection of NSCLC at an early stage is necessary for successful therapy and improved survival rates, because in most cases, patients have been at advanced stages at the time of diagnosis. A number of proteomic researches have been carried out, aimed to find some candidate biomarkers in biological samples which may be useful in early diagnosis of NSCLC [7–9].

Proteomic analysis was based on several techniques, including 2-DE, capillary electrophoresis (CE), multidimensional chromatography, and electrospray (ESI) and matrix assisted laser ionization/desorption (MALDI) mass spectrometric protein analysis coupling to protein and sequence databases. 2-DE is the most commonly used method to separate proteins in proteomic research. Although the 2-DE technology is capable of resolving large quantity of proteins, it has its limitation that can be troublesome, especially in proteomics applications, when the sample is relatively complex like urine and serum. When applying to urinary proteomics, it was

Abbreviations: AACT, alpha-1-antichymotrypsin; NSCLC, non-small cell lung carcinoma; PMSF, phenylmethylsulphonyl fluoride; DDH, high dihydrodiol dehydrogenase; ELISA, enzyme-linked immunosorbent assay; CE, capillary electrophoresis.

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doi:10.1016/j.jchromb.2010.10.026

found that it was hard to get a good 2-DE map, mainly due to the high concentrations of salts, metabolic wastes and small molecules urine [10]. In addition, the protein patterns on 2-DE maps can vary dramatically between either different individuals or different sample preparation methods. To avoid the troublesome use of isoelectric focusing (IEF) in 2-DE, in the present work, we have used 1D SDS-PAGE to separate the urinary proteins based on their molecular weights. Precipitation and ultrafiltration methods were employed to eliminate interfering materials and concentrate urinary proteins before 1D SDS-PAGE separation. Through these methods, five lung cancer related proteins were identified and the protein of interest was further validated both in urine and lung tissue.

2. Materials and methods

2.1. Urine collection and sample preparation

The human mid-stream urine specimens (first-voided urine in the morning) were collected from eight healthy donors (four males and four females, ages in the range of 55–63, who had not consumed aspirin or other non-steroidal anti-inflammatory drugs for at least 2 weeks. All females had no menstrual cycle at the time of collection) and three patients which were diagnosed with non-small cell lung cancer (adenocarcinoma cell type, stage IV, age range 55–65 years olds, without undergoing surgical resection or radio-therapy) at the first affiliated hospital of Chongqing Medical University. Informed consent was obtained from all donors. The period of urine specimen collection was from October 2009 to April 2010.

The urine samples were collected in the 50 mL polypropylene tubes. Immediately after collection, pooled normal urine samples from eight healthy donors and lung cancer urine samples from 3 patients were supplemented with protease inhibitors (1.67 mL of 100 mM NaN_3, 2.5 mL of 11.5 mM PMSF, and 50 μL of 1 mM leupeptin) to avoid proteolysis. The urine samples were stored on ice prior to centrifugation at 1500 × *g* for 10 min at 4 °C to remove insoluble solids. The precipitates were removed and the supernatants were stored at −80 °C to prevent bacterial growth. The protein concentration of the urine samples was measured using bradford method.

2.2. Protein precipitation and ultrafiltration

The thawed urine samples were diluted with ACN (1:5) and kept at 4 °C overnight for complete protein precipitation. The mixture was vortexed and centrifuged at 14,000 × *g* at 4 °C for 15 min. After washing with cold ACN, pellet was dried at room temperature to remove residual ACN.

The pellet was resuspended in 2 mL of multiple chaotropic sample solution (8 M urea, 2 M thiourea, 4% (w/v) CHAPS, 40 mM Tris base, 5 mM tributylphosphine and 10 mM acrylamide) and incubated at room temperature for 60 min to reduce and alkylate the proteins. The protein solution was centrifuged at 21,000 × *g* for 10 min at room temperature prior to desalting to remove non-soluble material. The protein solution was then placed on the Amicon Ultra-15 (5 kDa) centrifugal filter (Millipore, USA) and diluted with 12 mL of 8 M urea, 2 M thiourea and 4% (w/v) CHAPS. The protein solution was desalted and concentrated at 4000 × *g* at 4 °C until the volume reached 200 μL. Samples in the filter unit were collected and protein concentration was measured using bradford method.

2.3. 1D SDS-PAGE

Fifty micrograms urine samples were separated under denaturing conditions in a 4–12% polyacrylamide gel. Before separation on the gel, the urine samples were diluted in Laemmli buffer and boiled for 5 min. The SDS-PAGE gel was run in a Mini Protein 3 Cell (BIO-RAD, CA, USA) at 120 V for 2 h. After completion of the electrophoresis, the protein bands in the gel were visualized by Coomassie Blue staining and the image were acquired by an image scanner (BIO-RAD, CA, USA), which operated by the software Quality One (BIO-RAD, CA, USA).

2.4. In-gel digestion

The selected protein lanes on 1D SDS-PAGE were excised manually from the coomassie-stained gels, and transferred into 0.5 mL siliconized Eppendorf tubes before destained by incubation in 75 mM ammonium bicarbonate/40% ethanol (1:1). After destaining, the gel pieces were incubated in a solution of 5 mM DTT/25 mM ammonium bicarbonate (volume sufficient to cover the gel) at 60 °C for 30 min. The gel pieces were cooled to room temperature and the liquid was discarded. For alkylation of proteins, the gel was incubated in a solution of 55 mM iodoacetamide at room temperature for 30 min, and then the gel pieces were dehydrated in 100% ACN and dried. The gel pieces were then swollen in 10 mL of 25 mM ammonium bicarbonate buffer containing 20 mg/mL modified sequencing grade trypsin (Roche Applied Science) and incubated overnight at 37 °C. The tryptic peptide mixture was eluted from the gel with 0.1% formic acid.

2.5. LC–MS/MS analysis

Peptides were resuspended in 25 μL 0.1% formic acid and 20 μL was used for each LC–MS/MS analysis. An Agilent 1200 series nanoflow HPLC system (Agilent Technologies, Palo Alto, CA, USA) was run in the trapping mode with an enrichment column (560.3 mm, 5 mm particles) and a Zorbax 300SB C18 analytical column (150 mm × 0.075 mm, 3.5 mm particles). Sample was injected on the enrichment column via an autosampler. The mobile phase consisted of solvents A (water with 0.1% formic acid) and B (90% ACN, 10% water with 0.1% formic acid). The column was developed with a biphasic gradient of solvent B from 3% to 15% in solvent A in 2 min followed by an increase of B from 15% to 50% in 70 min. The column was regenerated by ten column volumes of 90% B followed by five volumes of 3% B. Both the enrichment and the analytical columns were submitted to the same development, washing and regeneration conditions. The total analysis time was 120 min, and the flow rate was fixed at 0.3 μL/min. ESI-MS and CID-MS/MS analyses were conducted on an Agilent 1100 Series LC/MSD Trap MS. The MS and MS/MS conditions employed were:

Drying gas flow: 4 L/min, 325 °C; capillary voltage: 1900 V; skim 1: 30 V; capillary exit: 75 V; trap drive: 85; averages: 1; ion current control: on; maximum accumulation time: 150 ms; smart target: 500,000; MS scan range: 300–2200; ultra scan: on.

MS/MS: number of parents: 5; averages: 1; fragmentation amplitude: 1.3 V; SmartFrag: on, 30–200%; active exclusion: on, 2 spectra, 1 min; prefer +2: on; exclude +1: on, MS/MS scan range: 200–2000; ultra scan: on; ion current control target: 500,000.

Due to statistical fluctuations of peptide precursor selection during MS/MS acquisition, three LC–MS/MS assays were run with each sample in order to be able to do a proper proteome comparison.

2.6. Protein identification and data analysis

Peptide and protein identifications were run automatically with the Spectrum Mill Proteomics Workbench Rev A.03.03.078 software from Agilent Technologies. Peak lists were created with the Spectrum Mill data extractor program with the following parameters: scans with the same precursor ±1.4 *m*/*z* were merged within a time frame of ±15 s; precursor ions needed to have a minimum S/N of 25; charges up to a maximum of 7 were assigned to

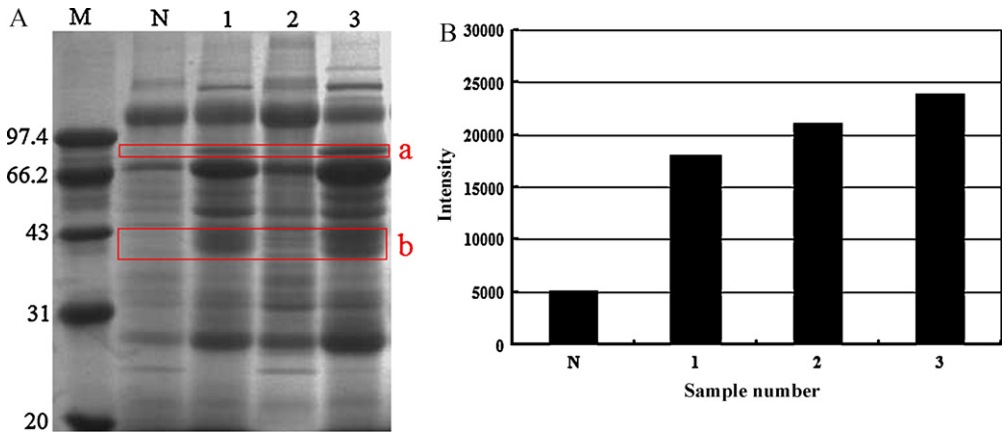

Fig. 1. 1D SDS-PAGE analysis of pooled normal urine and three NSCLC urine samples. (A) Fifty micrograms of urinary proteins was loaded on the gel for visualization by Coomassie Blue staining. M, protein marker; N, pooled normal urine sample; 1, 2, 3, three NSCLC urine samples. (B) Intensity of bands b in the gel was quantified by Quantity One imaging system.

Table 1
A list of the identified proteins.

Protein name	Protein ID	Score	% AA Coverage	Sample source	Subcellular location
Zinc-alpha-2-glycoprotein	IPI00166729	126.08	39	All	Secreted
Putative uncharacterized protein ALB	IPI00022434	125.50	23	N/2	Cytoskeleton
Actin, cytoplasmic 2	IPI00021440	118.92	22	N	Cytoplasmn
cDNA FLJ34642 fis, clone KIDNE2016918, highly similar to uromodulin	IPI00640271	115.33	12	All	Not classified
Orosomucoid 1	IPI00884926	99.68	36	All	Secreted
Protein AMBP, adrenomedullin binding protein	IPI00022426	87.37	21	N/2/8/24	Secreted
Keratin, type II cytoskeletal 1	IPI00220327	84.95	11	All	Membrane
Haptoglobin	IPI00641737	83.20	13	2/8/24	Secreted
Beta-2-glycoprotein 1	IPI00298828	81.94	27	2/8/24	Secreted
Matrix-remodeling-associated protein 8	IPI00153049	72.57	13	N/8	Membrane
Fibrinogen alpha chain	IPI00021885	71.74	9	All	Secreted
Aminoacylase-1	IPI00009268	58.37	17	N/8	Membrane
Clusterin	IPI00400826	54.40	11	N/2	Secreted
Keratin, type I cytoskeletal 9	IPI00019359	50.99	10	N/24	Cytoskeleton
Semenogelin-2	IPI00025415	49.57	6	N	Secreted
Phosphoglycerate kinase 1	IPI00169383	48.90	17	N/8	Cytoplasm
Argininosuccinate synthase	IPI00020632	44.85	9	8	Cytoplasm
Alpha-1-antitrypsin	IPI00553177	41.98	12	N/2/8	Secreted
Kallikrein-1 (KLKB1)	IPI00383717	37.63	17	N	Not classified
Isocitrate dehydrogenase [NADP] cytoplasmic	IPI00027223	37.59	8	N/2	Cytoplasm,Peroxisome
Plasma serine protease inhibitor	IPI00007221	35.95	8	N	Secreted
Alpha-1-antichymotrypsin	IPI00550991	35.67	8	2	Secreted
cDNA FLJ55146, highly similar to Complement C4-B	IPI00418163	32.59	3	8	Not classified
Apolipoprotein A-IV	IPI00304273	29.94	10	N/8	Secreted
Glutaminyl-peptide cyclotransferase	IPI00003919	29.36	6	N/2	Not classified
Monocyte differentiation antigen CD14	IPI00029260	26.63	9	2/8	Membrane
Chondroitin sulfate proteoglycan 4	IPI00019157	25.10	0	N	Membrane
Gelsolin	IPI00026314	23.40	3	N/24	Secreted
Keratin, type I cytoskeletal 10	IPI00009865	21.39	4	N/24	Cytoskeleton
Glutamyl aminopeptidase	IPI00014375	16.89	1	8	Membrane
Betaine–homocysteine S-methyltransferase 1	IPI00004101	16.39	3	8	Cytoplasm
Nidogen-1	IPI00026944	14.61	2	8	Secreted
Galectin-3-binding protein (tumor-associated antigen 90K)	IPI00023673	14.01	2	2	Membrane
Leucine-rich alpha-2-glycoprotein	IPI00022417	12.88	2	2	Secreted
Putative uncharacterized protein DKFZp686J11235	IPI00426060	12.62	1	N	Not classified
Hepatitis A virus cellular receptor 2	IPI00651768	12.30	4	2	Membrane
Non-secretory ribonuclease	IPI00019449	12.22	10	2	Cytoplasm
Aspartate aminotransferase, cytoplasmic	IPI00219029	11.83	3	8	Cytoplasm
Fructose-bisphosphate aldolase B	IPI00218407	11.43	4	2	Not classified
Low affinity immunoglobulin gamma fc region receptor III-a isoform a precursor	IPI00640044	11.31	3	N	Not classified

the precursor ion and the ^{12}C peak was also determined by the Data Extractor; $[M+H]^+$ comprised between 450 and 4000, and a scan time comprised between 0 and 300 min was used. Peptides were automatically identified by the Spectrum Mill Proteomics Workbench Rev A.03.03.078 software using UniProtKB/Swiss-Prot protein database (Geneva, Switzerland) search for tryptic peptides with the restriction to *Homo sapiens*. A mass tolerance of ±2.5 Da for the precursor ions and a tolerance of ±0.7 Da for the

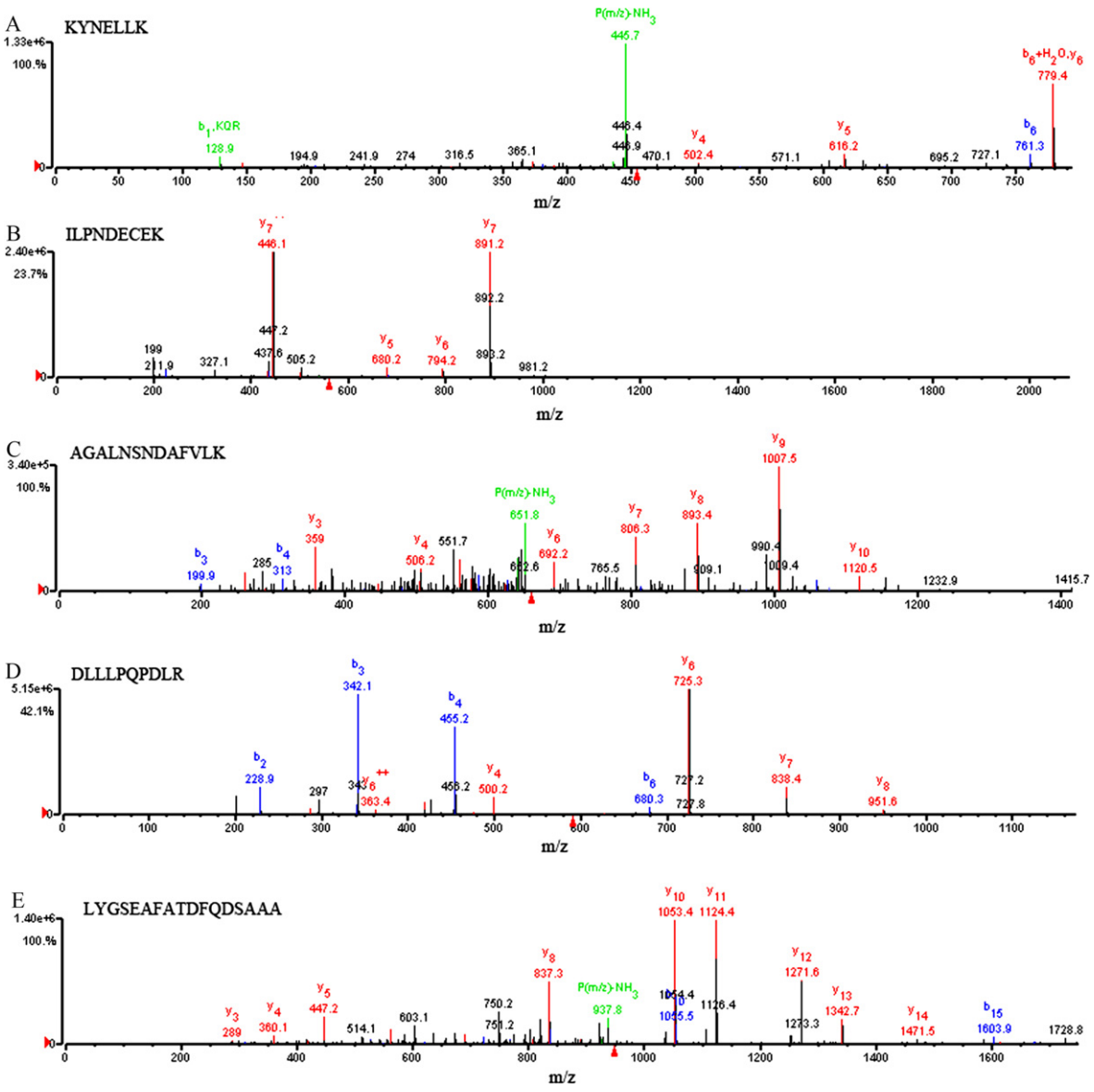

Fig. 2. The MS/MS map marked with b ions and y ions for 6 NSCLC associated proteins identified in urine. (A) MS/MS spectrum of peptide ion (MH+: 906.9927, *m*/*z* 454) from CLU clusterin isoform 1. (B) MS/MS spectrum of peptide ion (MH+: 1116.7927, *m*/*z* 558.9) from Intron-containing kallikrein (Fragment). (C) MS/MS spectrum of peptide ion (MH+: 1318.8127, *m*/*z* 659.91) from GSN Isoform 1 of Gelsolin precursor. (D) MS/MS spectrum of peptide ion (MH+: 1179.1327, *m*/*z* 590.07) from LRG1 Leucine-rich alpha-2-glycoprotein precursor. (E) MS/MS spectrum of peptide ion (MH+: 1890.7527, *m*/*z* 945.88) from SERPINA3 alpha-1-antichymotrypsin precursor.

fragment ions were used. Two missed cleavages were allowed. Carboxymethylated cysteines were set as fixed modification and oxidized methionine as variable modification. Spectrum Mill scores above 13 for complete proteins with a minimum score of 70% and a minimum scored peak intensity (SPI) for individual peptides were used as auto-validation criteria. These criteria are a good compromise between risking too many false positive if the values are set too low and the risk to miss real protein hits if the values are set too high. Auto-validation was necessary to ensure the same data analysis conditions for all the LC–MS/MS runs done in this work and to assure a fair comparison of the results.

2.7. Western blot

Fifty micrograms of proteins from urine was separated on 12% polyacrylamide gels. After electrophoresis, proteins were transferred to PVDF membranes (Millipore, USA). These blots were incubated for 2 h at room temperature in Tris-buffered-saline with Tween (20 mM Tris–HCl, 140 mM NaCl, pH 7.5, 0.05% Tween 20) containing 5% skim milk. After washing, the membranes were incubated with monoclonal mouse anti-AACT antibody (diluted 1:200, Abcam) overnight at 4 °C, then incubated with goat anti-mouse IgG antibody (diluted 1:200, Abcam) for 1 h at room temperature. ECL advance or plus western blot analysis system (Millipore, UK) was used to detect immunoreactive proteins.

2.8. Immunohistochemistry staining

The samples used for immunohistochemistry staining were formalin-fixed, paraffin-embedded tissues, which included 20 NSCLC patients and adjacent 20 normal lung tissues (16 male; 4 female; age, 52–62 years) at the first affiliated hospital of Chongqing Medical University. All samples were collected with informed consent.

Paraffinized tissue blocks were cut to obtain 5-μm-thick sections. Tissue sections on Super Frost slides were kept overnight at

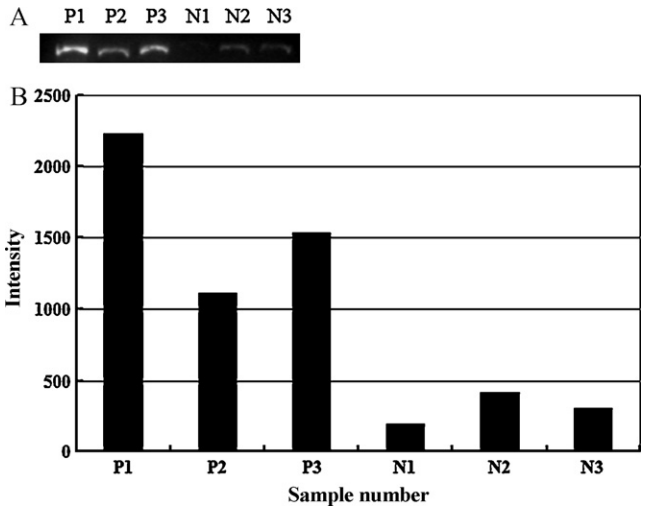

Fig. 3. Validation of AACT expression levels in urine by western blot analysis. Urine samples from three NSCLC patients and three normal age-matched individuals were used for western blot analysis. (A) representative western blot of urine using monoclonal anti-AACT antibody (diluted 1:200); P1 (++), P2 (+), P3 (++), proteins isolated from the NSCLC patients specimens; N1(−), N2(−), N3(−), proteins isolated from normal age-matched controls specimens. Significantly higher levels of AACT were found in urine from the NSCLC patients. (B) The bar plots demonstrate the AACT expression levels in the urine as detected by western blotting in the 20 NSCLC samples examined.

55 °C. Paraffin sections (5 μm) were deparaffinized and rehydrated in a series of xylene and ethanol baths of decreasing concentration. Deparaffinization and rehydratation were carried out in a series of xylene and ethanol baths of decreasing concentration. Endogenous peroxidase was inhibited by 3% hydrogen peroxide in PBS for 10 min. Antigen retrieval was performed by heating the sections in 0.01 M citrate buffer in a microwave oven. Nonspecific binding was blocked by incubating the tissue sections with 10% BSA in PBS for 60 min. Slides were then incubated overnight at 4 °C with monoclonal mouse anti-AACT antibody (Abcam) at a dilution of 1:200. Controls without primary antibodies were also included. After washing, a visualizing system containing secondary antibody, streptavidin–biotin, and DAB was used. Tissue samples were counterstained with hematoxylin, washed with 37 mM NH_4OH, and then covered. These steps were carried out in a wet chamber. Negative controls were performed by omitting the primary antibody.

Immunohistochemical staining level was based on the percentage of immunopositive levels according to the scoring guidelines: negative staining (∼10%), −; positive weak staining, +; positive strong staining, ++, respectively.

2.9. Statistical analysis

Statistical analysis was performed using the SPSS version 11.5 software package. A difference of $p < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. SDS-PAGE analysis

Fifty micrograms of urinary proteins were loaded on a gel for visualization. Fig. 1 shows the Coomassie Blue stained 1D SDS-PAGE of urinary proteins. As in the SDS-PAGE gel, based upon lane-by-lane comparisons, two bands showed significant differences in protein abundance between normal and lung cancer urines, marked a and b. Considering that low-molecular weight proteins (less than 40 kDa) can readily pass through the glomerular filtration barrier, thus are more likely to represent the situation of the circulation system, band b was chosen for further protein characterization.

3.2. Protein identification

After 1D SDS-PAGE analysis, we used Agilent nanoHPLC-chip-MS/MS ION TRAP 6330 to characterize differentially expressed proteins between normal persons and NSCLC patients in the bands. HPLC-Chip is a microfluidic chip-based device that can carry out nanoflow high performance liquid chromatography (HPLC). Compared with conventional column-based nanoflow HPLC, HPLC-Chip offers unparalleled ease of use, greater reliability and robustness and higher sensitivity. The low-energy collision CID-MS/MS data was then automatically searched against UniProtKB/Swiss-Prot protein database by Spectrum Mill Proteomics Workbench Rev A.03.03.078 software. There were about 786 MS/MS spectra generated, leading to the characterization of a total of 40 unique proteins. And their detailed information was listed in Table 1.

Among these proteins, there were five proteins related with lung caner, and their MS/MS spectrums were listed in Fig. 2. These proteins were:

3.2.1. Clusterin (CLU)

Several *in vitro* studies have examined the role of CLU in carcinogenesis, lung cancer progression, and response to chemo- and radiotherapy. Studies performed in lung cancer cell lines and animal models showed that CLU is up-regulated after exposure to chemo- and radiotherapy. A potential role proposed for the protein is cytoprotective. *In vivo*, a recent work analyzed the prognostic role of CLU in NSCLC, showing that CLU-positive patients with lung cancer had a better overall survival and disease-free survival than those with CLU-negative tumors. From the results of these studies, CLU has been hypothesized to represent a positive biomarker correlating with better overall survival in early-stage lung cancers [11].

3.2.2. Kallikrein 1 (KLK1)

Human tissue kallikreins are a family of 15 highly conserved serine proteases (KLK1–KLK15) encoded by the largest contiguous cluster of protease genes in the human genome. Kallikreins are directly or indirectly involved in cancer pathogenesis by promoting angiogenesis and degradation of extracellular matrix proteins [12]. By microarray analysis and real-time reverse transcription-PCR, a significant correlation between kallikreins overexpression and the squamous cell lung carcinoma histotype was observered compared with adjacent nonmalignant lung tissues [13]. Recently, a multiparametric panel of kallikrein biomarkers for lung cancer diagnosis in serum with relatively good accuracy has also been proposed [7].

3.2.3. Gelsolin

Gelsolin is a representative of actin-regulatory proteins with an 82 kDa mass and is present in most vertebrate tissues [14,15]. Sagawa and colleagues reported that gelsolin expression is reduced or not detected in various lung cancer cell lines and in more than half of the surgically resected tissues. Furthermore, they found that in PC10 cells, gelsolin suppressed the activation of PKCs involved in phospholipid signaling pathways in a human lung cancer cell line and inhibited cell growth and tumorigenicity in nude mice, arguing that the introduction of gelsolin into lung cancer might possibly serve as an antitumor gene therapy in the future [16].

3.2.4. Leucine-rich α-2-glycoprotein (LRG)

LRG is an approximately 50 kDa glycoprotein [17], and contains repetitive sequences with a leucine-rich motif [18]. LRG has been reported to be expressed by liver cells [19] and neutrophils [20], although its function remains unclear. Previous reports have shown

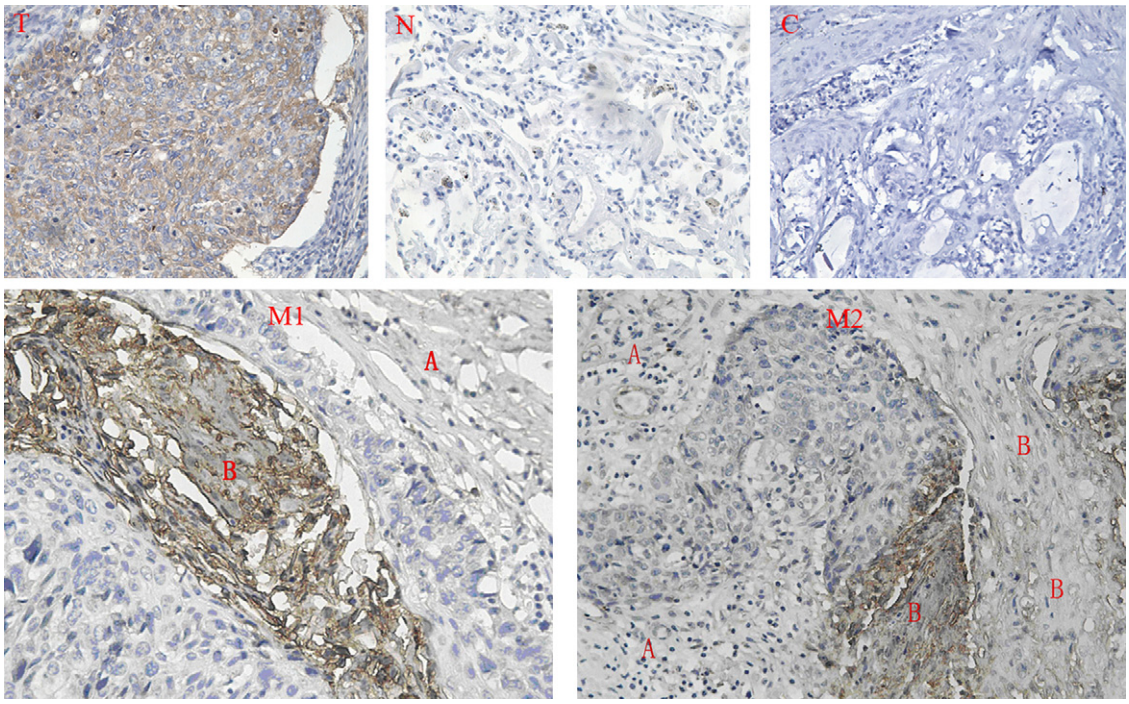

Fig. 4. mmunohistochemical staining for AACT in lung tissues. Paired NSCLC tumor tissue (T) and adjacent non-tumor lung tissue (N) from paraffin-embedded lung tissue sections were stained by anti-AACT antibody. Controls without primary antibody were also shown (C). In a tissue mixed block (M1, M2), region A represents non-tumor part, whereas region B represents tumor part, which is also consistent with that shown in non-tumor and tumor part.

Table 2
Immunohistochemistry analysis of relative AACT staining level between non-tumor and tumor region. "A" represents adenocarcinoma carcinoma, "S" represents squamous cell carcinoma.

Sample no.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Age	49	35	56	55	55	64	69	49	62	52	62	62	63	57	73	53	71	57	53	69
Gender	M	M	M	M	M	M	M	M	M	F	M	M	M	F	F	M	M	F	M	M
subtype	S	S	S	S	S	A	A	S	S	A	S	A	A	A	A	S	A	A	S	A
IHC result																				
Tumor	++	+	++	++	+	–	+	+	+	++	+	++	++	++	++	++	++	+	++	+
Non-Tumor	+	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

that serum LRG concentrations are increased in several types of cancers. Recently, LRG was detected at higher levels both in lung cancer sera and plasma compared to normal individuals [21,22].

3.3. Protein validation by western blot

To validate the LC–MS/MS data, we analyzed the levels of AACT in three additional healthy and NSCLC urine samples by western blot. AACT was not identified by LC–MS/MS analysis in normal urine samples (N) but detected in all urine samples by western blot, and the level of AACT was higher on the whole in the urine from three NSCLC patients compared to samples from normal healthy individuals (Fig. 3A).

3.4. Immunohistochemistry staining

To verify the expression level of AACT in lung tissues, immunohistochemistry staining was performed on paraffin-embedded tissue sections that contained both tumor tissue and adjacent non-tumor lung tissue from 20 NSCLC patients. As shown in Fig. 4, although the tumor tissues were strongly stained, the non-tumor tissue regions were less stained. Antibodies against AACT stained tumor cells with a cytoplasmic pattern, whereas non-tumor cells exhibited negative staining. In tissue block containing both tumor and adjacent non-tumor region, AACT was strongly stained in tumor tissues (region B) compared with non-tumor tissue (region A) as shown in Fig. 4 M1, M2. Totally, 18 out of 20 cases (90%) were more stained than in adjacent tumor tissue relative to non-tumor (3 out of 20 cases, 15%) (shown in Table 2). The result showed significantly increased AACT level in lung cancer compared with normal lung tissue.

AACT is a serine proteinase inhibitor produced by various tumor cells and thought to be association with tumorigenicity in patients with various types of cancer [23]. By RT-PCR, western blotting, and immunohistochemical methods, AACT synthesis were confirmed in several lung adenocarcinoma cell lines and surgically resected lung adenocarcinomas tissues. The data in clinical materials, combined with results of the previous report that AACT in breast cancer acts as a minor growth factor-like substance, suggested that AACT expression in lung adenocarcinoma also may be associated closely with tumor progression and especially with tumor growth [24]. Combined with our finding, we strongly proposed AACT to be a candidate biomarker for objective and non-invasive diagnosis of NSCLC in urine.

4. Conclusions

In summary, using a comparative proteomic discovery approach combined 1D SDS-PAGE and nano-HPLC-chip-MS/MS, we identified and validated increased tissue and urinary AACT levels in

NSCLC patients. Many others previously reported NSCLC-associated proteins were also identified in our study, which added further support of this approach for screening of novel biomarkers. As our cohort is relatively small, further large scale clinical investigation would be carried out to explore the applicability of such markers in clinical practice and additional validation of other biomarkers may be improved.

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