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

Yuan Zhang [a], Yanyan Li [a], Feng Qiu [b], Zongyin Qiu [a,*]

[a] College of Pharmacy, Chongqing Medical University, Chongqing 400016, China
[b] First Affiliated Hospital, Chongqing Medical University, Chongqing, China

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.

© 2010 Elsevier B.V. All rights reserved.

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.

* Corresponding author. Fax: +86 023 68485277.
E-mail addresses: cheungyang@hotmail.com, 2007yuan@gmail.com (Z. Qiu).

1570-0232/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
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

Download English Version:

https://daneshyari.com/en/article/1213969

Download Persian Version:

https://daneshyari.com/article/1213969

Daneshyari.com