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REGULAR ARTICLE

Different techniques for urinary protein analysis of normal and lung cancer patients

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Many components in urine are useful in clinical diagnosis and urinary proteins are known as important components to define many diseases such as proteinuria, kidney, bladder and urinary tract diseases. In this study, we focused on the comparison of different sample preparation methods for isolating urinary proteins prior to protein analysis of pooled healthy and lung cancer patient samples. Selective method was used for preliminary investigation of some putative urinary protein markers. Urine samples were passed first through a gel filtration column (PD-10 desalting column) to remove high salts and subsequently concentrated. Remaining interferences were removed by ultrafiltration or four precipitation methods. The analysis of urinary proteins by high-performance liquid chromatography and sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed many similarities in profiles among preparation methods and a few profiles were different between normal and lung cancer patients. In contrast, the results of two-dimensional gel electrophoresis (2-DE) showed more distinctly different protein patterns. Our finding showed that the sequential preparation of urinary proteins by gel filtration and ultrafiltration could retain most urinary proteins which demonstrated the highest protein spots on 2-D gels and able to identify preliminary urinary protein markers related to cancer. Although sequential preparation of urine samples by gel filtration and protein precipitation resulted in low amounts of proteins on 2-D gels, high M_r proteins were easily detected. Therefore, there are alternative choices for urine sample preparation for studying the urinary proteome and identifing urinary protein markers important for further preclinical diagnostic and therapeutic applications.

Keywords:
Lung cancer / Sample preparation / Two-dimensional gel electrophoresis / Urinary protein markers / Urine

1 Introduction

Human biological fluids contain various types of markers and these biomarkers are useful for prognosis, diagnosis and drug development for many diseases. Urine is a human sample that is easily obtained in clinical diagnosis. Some components in urine were used to analyze different patterns between normal and cancer patients such as nucleosides by RP-HPLC [1, 2] and CE [3], and for monitoring urinary tamoxifen and its metabolites in breast cancer treatment [4]. There are many ongoing studies looking for disease-related biomarkers in urine because urinary protein is one of the important components that can define a diseased state.

In the present study, many proteomic techniques were used to investigate urinary protein markers. Proteome maps of human normal urinary proteins were constructed to identify proteins normally found in urine [5–7]. There are few studies which use urine to compare normal and diseased

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Abbreviations: DTE, dithioerythreitol; **GM2AP**, GM2 activator protein; **TTR**, transthyretin

proteomics techniques to find out some urinary protein markers from normal and diseased states such as inflamed pilonidal abscess diseased [8] and renal diseased [9]. In bladder cancer, a putative urinary marker was used for the follow-up of patients [10]. Sample preparation of urine samples is the most important aspect of data generation. Specifically, high concentrations of salts, metabolic wastes and some small molecules must be removed before analysis. Dialysis is commonly used to desalt urine samples, followed by other protein concentration methods, but requires more than 2 days. Ultracentrifugation [6] and solid phase extraction [8] are precipitation methods also used to prepare urine samples.

In this study, we used different techniques for preparation of urine samples prior to protein analysis. To prepare urine samples a PD-10 desalting column was first used to remove high concentrations of salts. This was followed by ultrafiltration or precipitation methods. Then, the prepared urine samples were examined by different analysis methods in order to (i) compare the urine preparation methods, (ii) compare the urinary protein contents from human normal and lung cancer patients, and (iii) investigate urinary protein markers of lung cancer. Thus, we present a method for urine sample preparation prior to protein analysis that allows removal of interfering compounds but retains high protein amounts. Furthermore, we found some preliminary urinary protein markers for lung cancer which may become useful markers for diagnostic and pharmaceutical applications.

2 Materials and methods

2.1 Materials

Ammonium bicarbonate, urea, CHAPS and SDS were from J. T. Baker (Phillipsburg, NJ, USA). Polycrylamide was from Amresco (Solon, OH, USA). Sodium azide and ACN were from ACROS Organics (New Jersey, USA). Acetone, chloroform and TFA were from Sigma (St. Louis, MO, USA). Methanol, chloroform and TCA were from Merck (Darmstadt, Germany). Dithioerythreitol (DTE) was from Appli-Chem (Darmstadt, Germany). Iodoacetamide was from Fluka (Buchs, Switzerland). Sypro Ruby was from Molecular Probes (Eugene, OR, USA). Deionized water was prepared with a tandem Milli-Q system (Millipore, Bedford, MA, USA) and used for the preparation of all buffers.

2.2 Preparations of human urine samples

Over 200 mL of clean catch first urine samples were collected in the morning from ten healthy donors and five lung cancer patients with non-small cell lung cancer (adenocarcinoma cell type, stage IV, age range 40–50 years olds). Urine samples collected in separated polypropylene tubes (NUNC, Rokilde, Denmark) containing sodium azide were stored at −80°C until used. Pooled normal urine samples from ten healthy donors and pooled lung cancer urine samples from five patients were supplemented with protease inhibitor cocktail to avoid proteolysis. The samples were centrifuged at 10 000 *g* for 20 min to remove insoluble solids. The supernatants were first passed through a PD-10 desalting column (Amersham Biosciences, Uppsala, Sweden) and eluted with 10 mM phosphate buffer, pH 7.5. The fractions containing proteins were collected and lyophilized.

2.2.1 Ultrafiltration

Lyophilized urine samples were resuspended in 10 mM phosphate buffer, pH 7.5 and loaded onto centricon 10 kDa membrane (Millipore) in order to concentrate the proteins and remove small interference molecules. Briefly, the centricons were spun at 8000 *g*, at 4°C for 1 h. The same buffer was added until the conductivity of the salts in the samples was less than 100 mS/m. The samples were then lyophilized.

2.2.2 Acetone precipitation

Lyophilized urine samples were resuspended in 10 mM phosphate buffer, pH 7.5 and two volumes of cold acetone was added. The mixture was stored overnight at −20°C and a pellet was obtained by centrifugation at 10 000 *g* at 4°C for 15 min. The pellet was vacuum dried.

2.2.3 ACN/TFA precipitation

Lyophilized urine samples were resuspended in 10 mM phosphate buffer, pH 7.5 and added with two volumes of cold ACN/0.1% TFA [11]. The mixture was vortexed and centrifuged at 10 000 *g* at 4°C for 15 min. The supernatant was removed and the pellet was vacuum dried.

2.2.4 Methanol/chloroform/water precipitation

Lyophilized urine samples were resuspended in 10 mM phosphate buffer, pH 7.5 and an aliquot of urine sample (0.1 mL) was added to 0.4 mL of methanol. The mixture was vortexed and centrifuged for 10 s at 9000 *g*. The mixture was added to 0.1 mL of chloroform, vortexed and centrifuged again for 10 s at 9000 *g*. Water (0.3 mL) was added and the sample was vortexed vigorously and centrifuged for 1 min at 9000 *g*. The upper phase was carefully removed and discarded. A further 0.3 mL of methanol was added into the rest of the chloroform phase and the interphase with the precipitated protein. The sample was mixed and centrifuged again for 2 min at 9000 *g*. The supernatant was removed and the protein pellet was vacuum dried [12].

2.2.5 TCA/acetone precipitation

Lyophilized urine samples were resuspended in 10 mM phosphate buffer, pH 7.5 and two volumes of cold 20% TCA in acetone (−20°C) was added. The mixture was stored overnight at −20°C and a pellet was obtained by centrifugation at

10 000 *g*, 4°C for 15 min. The pellet was washed twice: first with cold acetone containing 20 mM DTE and then with cold acetone without DTE, and centrifuged as described above. The supernatant was removed and the pellet was vacuum dried.

2.3 Protein analysis methods

2.3.1 RP-HPLC

Analysis of pooled urine samples were performed using a reverse-phase HPLC column (4.6 × 250 mm, Nucleosil 7C18) attached to a L-4250 UV-VIS detector and an L-7100 pump (all from Hitachi, Tokyo, Japan) with a 20 μL sample injection loop. Two mobile phases, A (0.1% v/v TFA, 5% v/v ACN) and B (0.1% v/v TFA, 95% v/v ACN), were used for all samples. All mobile phases were filtered (45 μm filter for organic solvent; Millipore) and degassed by sonication (Bransonic 52; Branson, Shelton, CT, USA). All solvents were HPLC grade. Each urine sample was resuspended in mobile phase A at a concentration of 1 mg/mL, filtered and injected into the HPLC column with a final volume of 10 μL. A linear gradient of 5% ACN to 95% ACN (0–100% mobile phase B) was used for 25 min within a 30 min running time at a flow rate of 1 mL/min. Protein was monitored by measuring UV absorption at 280 nm.

2.3.2 SDS-PAGE

Urine samples were separated under denaturing conditions in a 4–20% polyacrylamide slab gel (Invitrogen, Leek, The Netherlands). The lyophilized urine samples were resuspended in sample buffer (50 mM Tris-HCl, pH 6.8, 0.1 M DTT, 10% glycerol, 2% SDS, and 0.1% bromophenol blue) at a concentration of 1 mg/mL and heated at 95°C for 5 min. Each urine sample solution (10 μg/well) was separately loaded into gel wells. The SDS-PAGE gel was run in a Novex Xcell II (Novex, SanDiego, CA, USA) at 20 mA *per* gel. After completion of electrophoresis, the protein bands in the gel were visualized by Sypro Ruby staining and scanned by using a Typhoon 9200 image scanner (Amersham Biosciences). The Low Molecular Weight Calibration Kit (Amersham Biosciences) was used as standard M_r marker proteins.

2.3.3 2-DE

Lyophilized urine samples were dissolved in lysis buffer (7 M urea, 4% CHAPS, 4 mM Tris base, 2 M Thiourea, 2% IPG buffer pH 3–10 nonlinear, 65 mM DTE). Each sample was sonicated, centrifuged then applied onto IPG strips (18 cm, pH 3–10 nonlinear; Amersham Biopsciences) with a final protein concentration of 300 μg in 350 μL determined by the Coomassie protein assay, according to the Bradford protein dye binding assay [13]. IPGphor IEF (Amersham Biosciences) was performed under the following condition: IPG strips were rehydrated passively and/or actively for 12 h at 30 V followed by ramping to 100 V for 1 h, 250 V for 1 h, 500 V for 1 h, 1000 V for 1 h, 2000 V, 1 h, and focusing at 6000 V for up to 50 000 Vh. After IEF, the IPG strips were equilibrated in equilibration buffer 1 (50 mM Tris-HCl, pH 8.8, 6 M urea, 30% v/v glycerol, 2% w/v SDS, 2% w/v DTE and a trace of bromophenol blue) for 15 min, and then subsequently alkylated in buffer 2 (50 mM Tris-HCl pH 8.8, 6 M urea, 30% v/v glycerol, 2% w/v SDS, 2.5% w/v iodoacetamide and a trace of bromophenol blue) for 15 min. Each equilibrated IPG strip was placed on top of the 15% polyacrylamide gel (18 × 18 cm) and covered with 0.5% agarose. The second-dimensional separation was carried out at 40 mA *per* gel at 15°C until the bromophenol blue dye front reached the bottom of the gel. At the end of each run, the 2-D gels were stained with Sypro Ruby and scanned using a Typhoon 9200 image scanner (Amersham Biosciences). In addition, the 2-D gel images were exported to the image analysis software program, PDQuest 2-D analysis software version 7.1.1 (Bio-Rad, Hercules, CA, USA).

2.3.4 Protein digestion

Protein spots were manually excised from the gels and transferred to 500 μL siliconized Eppendorfs. The gel pieces were washed twice with 200 μL of 50% ACN/25 mM ammonium bicarbonate buffer, pH 8.0, for 15 min each. The gel pieces were then washed once with 200 μL of 100% ACN and dried using a Speed-Vacuum concentrator. Dried gel pieces were swollen in 10 μL of 25 mM ammonium bicarbonate containing 0.1 μg trypsin; (sequencing grade; Promega, Madison, WI, USA). Gel pieces were then crushed with a siliconized blue stick and incubated at 37°C for at least 16 h. Peptides were subsequently extracted twice with 50 μL of 50% ACN/5% TFA, then the extracted solutions were combined and dried using a SpeedVac concentrator. The peptides or pellets were then resuspended in 10 μL of 0.1% TFA and the suspended solutions were purified using ZipTip C18 (Millipore). Ten microlitres of sample was drawn up and down in the ZipTip 10 times and the ZipTip was washed with 10 μL of 0.1% formic acid by drawing up and expelling the washing solution three times. The peptides were eluted with 5 μL of 75% ACN/0.1% formic acid.

2.3.5 MALDI-MS and MS/MS analysis

For MALDI MS and MS/MS analysis, the samples were premixed in a ratio of 1:1 with matrix solution (5 mg/mL CHCA in 50% ACN, 0.1% v/v TFA and 2% w/v ammonium citrate) and spotted onto the 96-wells format MALDI sample stage. Data was directed acquisition on the Q-TOF Ultima MALDI instrument which was fully automated with predefined probe motion pattern and the peak intensity threshold for switching over from MS survey scanning to MS/MS, and from one MS/MS to another. Within each well, as many parent ions meeting the predefined criteria (any peak within the *m/z* 800–3000 range with intensity above 10 count

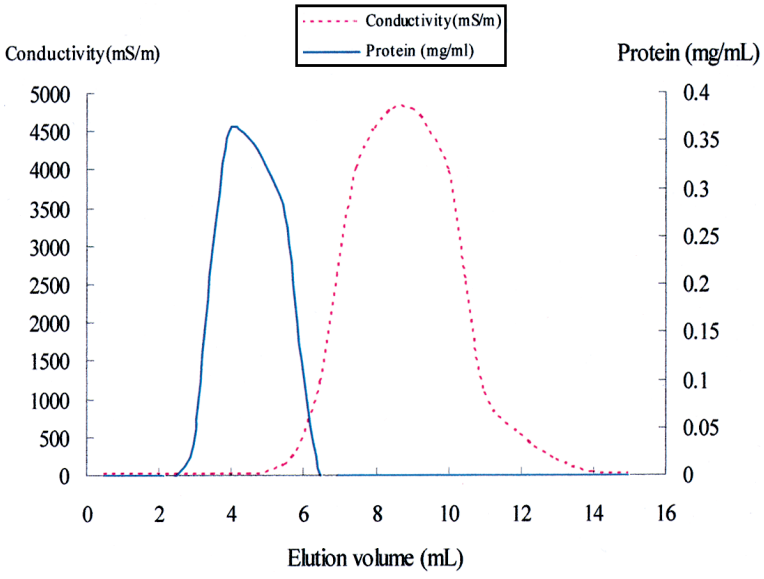

Figure 1. Separation of urinary proteins by Sephadex gel filtration (PD10 column; Amersham Biosciences). After 2.5 mL of urine samples were loaded into the column, urinary proteins were eluted with 10 mM phosphate buffer, pH 7.5. All eluted protein fractions were collected (0.5 mL/fraction). Red and blue lines represent the conductivity and protein contents, respectively. Protein fractions 3–6 were pooled and concentrated by lyophilization.

± include/exclude list) were selected from the most intense peak for CID MS/MS using argon as the collision gas and a mass dependent ± 5 V rolling collision energy until the end of the probe pattern was reached (all details are available at http://proteome.sinica.edu.tw). The MASCOT MS/MS ion search program (http://www.matrixscience.com) was used for peptide sequence searching. Search parameters allowed for oxidation of methionine, carbamidomethylation of cysteine and one missed trypsin cleavage were selected for searching the Swiss-Prot database. Protein identification was repeated at least once using spots from different gels. In addition, the identified proteins were searched for the description based on Swiss-Prot and NCBI protein databases.

3 Results and discussion

3.1 Urine sample preparation

It is well known that urine contains trace amounts of protein originating from blood plasma, the kidneys and the urogenital tract, and amounts of body filtrates such as water, salts, electrolytes and nitrogenous waste products [14, 15]. Thus, the sample preparation of urine samples is very important for prefractionation, protein analysis and preliminary screening of biomarkers. For these reasons, we used different sample preparations to prepare urine samples, which were pooled together to reduce variation of individual samples and composition, prior to analysis. First, the high abundance of interfering molecules containing salts, electrolytes and/or some nitrogenous waste products in urine samples were removed by Sephadex gel filtration using a PD-10 desalting column (Fig. 1) because they could negatively affect further proteomic analysis. Although the major urinary proteins were distinctly separated from small interferences in the elution volume of 3–6 mL, the urine samples still contained some salts from the elution buffer and some interfering substances. However, the first step of urine sample preparation by gel filtration removed most of the salts and other electrolytes from the urine samples. After that, the proteins in the urine samples were concentrated and the remaining salts and some nitrogenous waste products were removed by various preparation methods in order to reduce interfering molecules for further analysis, especially in the first dimension of 2-DE. Ultrafiltration and four precipitation methods were compared to find the best sample preparation of urine samples for investigating urinary protein markers in lung cancer by high-throughput analysis techniques.

3.2 RP-HPLC analysis

The components in urine samples from normal and lung cancer patients prepared by different preparation methods were separated by RP-HPLC and demonstrated different chromatogram profiles (Fig. 2). RP-HPLC chromatograms from all prepared urine samples showed a major protein peak in an elution range of 40–80% ACN, indicating that the urine components are dependent on hydrophobic properties. Sample preparations of normal urine and lung cancer urine by ultrafiltration, acetone precipitation and ACN/TFA precipitation showed similar profiles (Fig. 2: A1–3, B1–3). In contrast, samples prepared by methanol/chloroform/water and TCA in acetone precipitations showed less protein components and some peaks disappeared (Fig. 2: A4–5, B4–5). In addition, the chromatogram profiles of normal and lung cancer urines prepared by each preparation method were compared by superimposing the traces and showed distinct differences in protein components, especially at 40–80% ACN elution (Fig. 2, C1–C5). By this analysis, we found that different sample preparation methods for urine samples provided different protein profiles. Moreover, RP-HPLC analysis tended to high light different urinary proteins between normal and lung cancer that may be biomarkers of lung cancer disease.

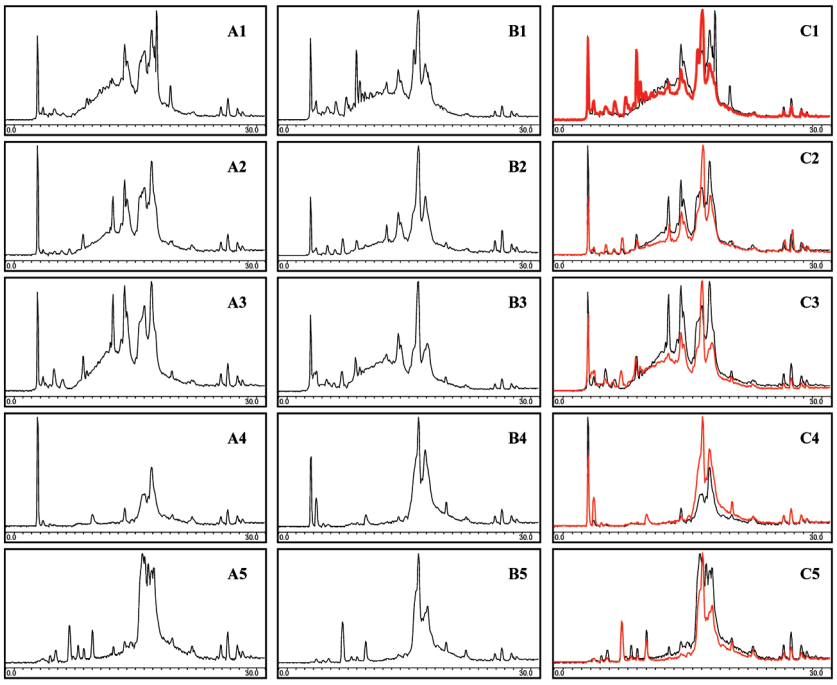

Figure 2. RP-HPLC chromatograms of pooled normal (A) and lung cancer (B) urine samples preparing by ultrafiltration, acetone precipitation, ACN/TFA precipitation, methanol/chloroform/water precipitation and TCA in acetone precipitation. The overlapped chromatogram profiles of urine samples (black line) from normal and lung cancer (red line) patients is shown in (C). The samples were analyzed on a Nucleosil 7C18, 4.6 × 250 mm column using a linear gradient of 0–100% mobile phase B at a flow rate of 1 mL/min for 25 min and washed with 100% mobile phase B until the running time reached 30 min. Mobile phase A was 5% v/v ACN, 0.1% v/v TFA and mobile phase B was 95% v/v ACN, 0.1% v/v TFA. Protein elution was monitored at 280 nm absorbance. 1, ultrafiltration; 2, acetone precipitation; 3, ACN/TFA precipitation; 4, methanol/chloroform/water precipitation; 5, TCA/acetone precipitation.

3.3 SDS-PAGE analysis

A SDS-PAGE gel of urine samples showed no differences in protein patterns for each sample preparation method but showed the different protein patterns between normal and lung cancer urines (Fig. 3). The major differences between lung cancer urine samples compared to normal urine samples were observed at 14 kDa and in the range of 28 to 42 kDa where protein bands with higher intensity than in normal urine samples were found. In addition, lung cancer urine samples had less protein at 50 kDa than normal urine samples. Although SDS-PAGE could not show the major differences between sample preparations because of limited separation in one dimension, it showed some different protein bands between normal and lung cancer disease.

3.4 2-DE analysis

The 2-DE images of normal and lung cancer urine samples prepared by different preparation methods are demonstrated in Fig. 4. The 2-DE image of urine samples prepared by ultrafiltration showed the highest protein spots both in normal and lung cancer patient samples compared to theirs images resulting from precipitations methods. One factor that influences separation by 2-DE analysis is the conductivity of the samples. Each preparation method gave different conductivities as shown in Table 1. TCA precipitation provided the lowest conductivity and ultrafiltration was manually controlled for removing salts. However, the effect of salts on 2-DE is reduced when the conductivity of samples is lower than 200 mS/m (20 mM NaCl) or when using IEF paper (Amersham Biosciences) to absorb excess salts. Using precipitation methods, some protein spots were lost and the protein spots on 2-D gels were poorly focused, especially at the M_r ranger lower than 30 kDa. Urine samples from normal and lung cancer patients prepared by methanol/chloroform/water precipitation had the lowest amount of protein in the low M_r range, but had many protein spots in the M_r range higher than 60 kDa (Fig. 4: A4, B4). Using the ultrafiltration

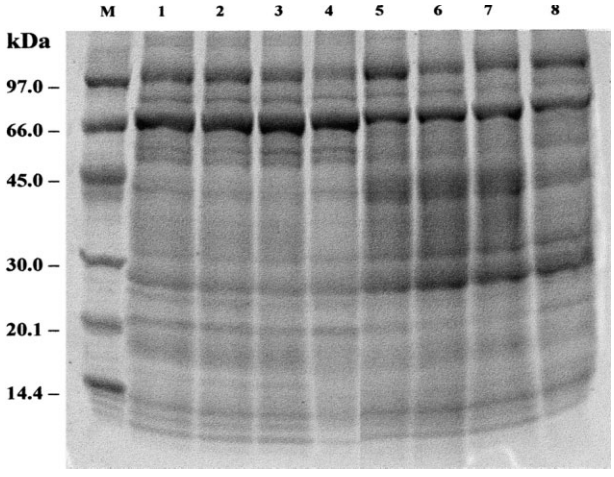

Figure 3. SDS-PAGE of pooled urine samples from normal and lung cancer patients. SDS-PAGE was carried out on a mini 4–20% gradient gel with 10 μg protein/well. Lane M is the protein standard markers, lanes 1–4 are pooled normal urine samples prepared using ultrafiltration, acetone precipitation, ACN/TFA precipitation and methanol/chloroform/water precipitation, respectively. Lanes 5–8 are pooled lung cancer urine samples prepared by using ultrafiltration, acetone precipitation, ACN/TFA precipitation and methanol/chloroform/water precipitation, respectively.

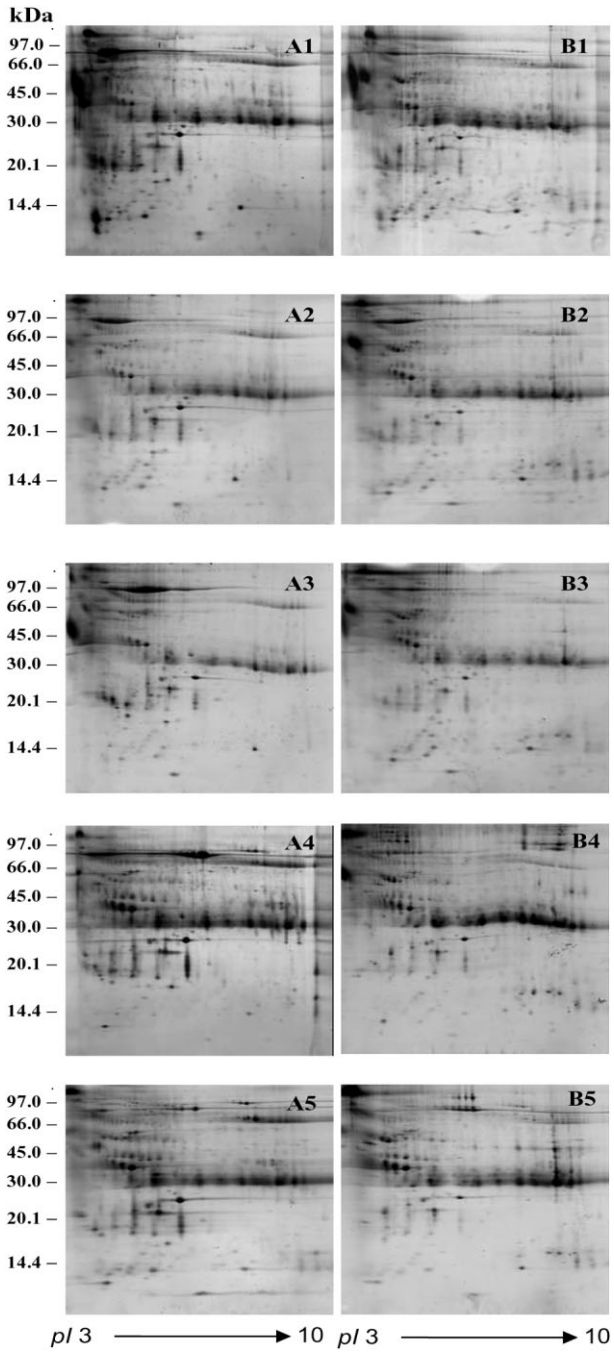

Figure 4. 2-DE gel images of pooled normal urine samples (A) and lung cancer urine pooled samples (B), prepared by different methods. Gels A1 and B1 represent urine samples prepared using ultrafiltration of pooled normal and lung cancer patients, respectively. Gels A2 and B2 represent urine samples prepared using acetone precipitation of pooled normal and lung cancer patients, respectively. Gels A3 and B3 represent urine samples prepared using ACN/TFA precipitation of pooled normal and lung cancer patients, respectively. Gels A4 and B4 represent urine samples prepared using methanol/chloroform/water precipitation of pooled normal and lung cancer patients, respectively. Gels A5 and B5 represent urine samples prepared using TCA in acetone precipitation of pooled normal and lung cancer patients, respectively.

Table 1. Summary of preparation methods for urine samples from normal and lung cancer patients

Sample preparation methods	Conductivity (mS/m)	Protein spots on 2-D gel image	
		Normal urine	Lung cancer urine
Ultrafiltration	<100 mS/m	829	827
Acetone precipitation	>100 mS/m	467	547
ACN/TFA precipitation	>100 mS/m	393	458
Methanol/chloroform/water precipitation	>100 mS/m	784	611
TCA/Acetone precipitation	15–20 mS/m	518	518

The conductivity of each sample was detected followed by analysis with 2-DE which detected the quantity of protein spots by PDQuest 2-D analysis software, version 7.1.1 (Bio-Rad).

method allowed most protein components in urine samples to be visualized on 2-D gels with good separation and higher protein intensity than precipitation methods which have denaturing conditions due to the organic solvents used. Although organic solvents, such as acetone, methanol and ethanol can easily precipitate the hydrophilic proteins, hydrophobic proteins tend to remain soluble in the aqueous fraction [16]. In addition, some urinary proteins have high solubility in solution according to the source of the urine sample that most of the proteins dissolved in water; therefore, some proteins are difficult to precipitate by organic solvent.

Furthermore, the quantity of urinary protein spots between normal and lung cancer urines were analyzed by PDQuest 2-D analysis software version 7.1.1. The total number of spots detected is shown in Table 1. Both urine samples of normal and lung cancer patients prepared by ultrafiltration showed the highest number of protein spots on 2-D gel images while the urine samples prepared by ACN/TFA precipitation showed the lowest. In contrast, the number of urinary protein spots on 2-D gels detected in our work was higher than those obtained in previous research in which urine samples were prepared by acetone precipitation and ultracentrifugation [6], solid-phase extraction [8], and dialysis followed by ultrafiltration [9]; except for the work of Pieper *et al.* [7] who reported a similar protein separation profile. The above reports used only one or a few methods of urine preparation for urinary protein analysis. Their protein analysis results showed poor protein resolution in separations. Thus, we compared sample preparations of urine samples and showed the importance of good sample preparation for subsequent protein analysis. Moreover, we used the selective preparation for investigating some urinary protein markers in lung cancer disease. The PDQuest software was also used to compare the protein patterns of urine samples in both normal and lung cancer patients (Fig. 5). According to the result above, we chose 2-D gel images of

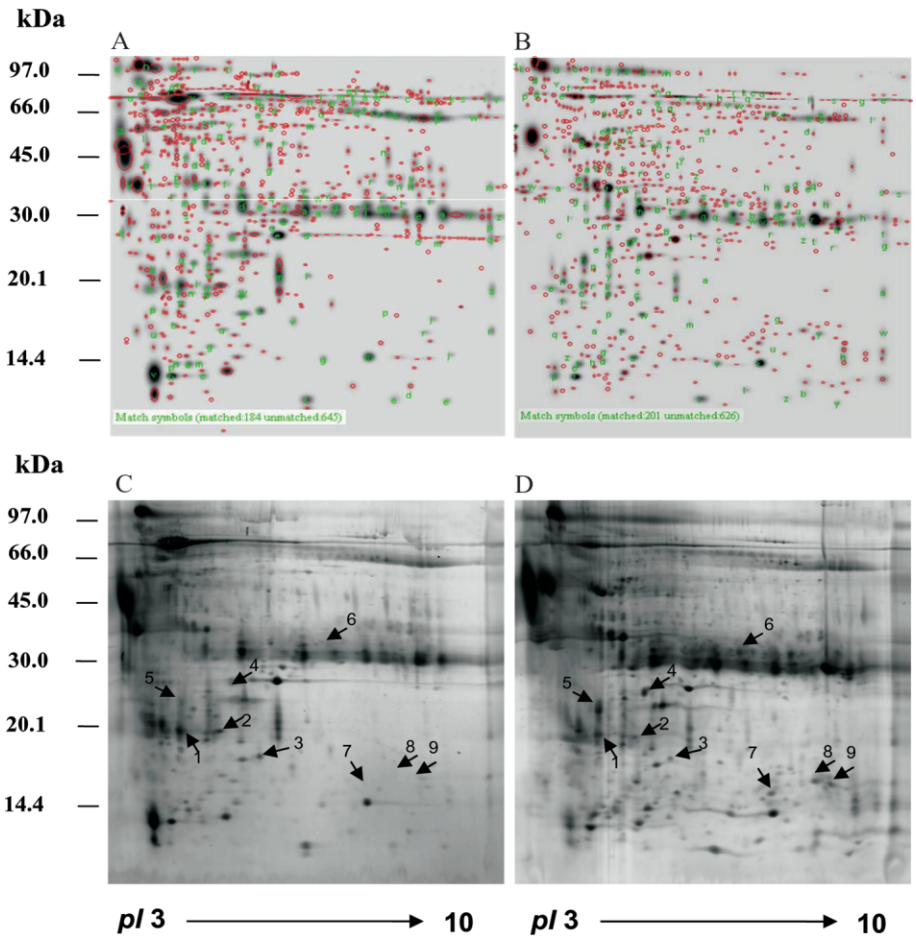

Figure 5. Comparison of 2-D gel images of pooled normal and lung cancer urine samples prepared by ultrafiltration and analyzed with PDQuest 2-D software version 7.1.1. Gels: (A) normal urine as the reference gel compared to lung cancer urine. (B) lung cancer urine as the reference compared to normal urine. (C) Normal urinary proteins. (D) Lung cancer urinary proteins. Green labeled spots represent matched spots and red labeled spots represent unmatched spots. The black arrows show proteins that were excised and analyzed by MALDI Q-TOF MS.

normal and lung cancer urine samples sequentially prepared by gel filtration and ultrafiltration to compare protein patterns and identify proteins because this preparation gave a good separation on 2-DE and provided the highest number of protein spots. The majority of differentially expressed proteins were in the M_r range below 30 kDa which was similar to the results from SDS-PAGE. Nine spots showing differential expression in both normal and lung cancer were excised from gels, digested with trypsin and identified by MALDI Q-TOF MS or MS/MS analysis.

3.5 Protein identification

Nine major protein spots on 2-D gels were analyzed by MALDI-TOF-MS or MS/MS and used in searches for matching proteins and their functions (Table 2 and Table 3). All recognized protein spots in the 2-D gel were identified by PMF. In all cases, the spots were sequentially subjected to MS/MS sequencing to identify the proteins. We found that there were three down-regulated proteins and six up-regulated proteins, related to lung cancer disease. The down-regulated proteins are CD59 glycoprotein precursor, activator of cAMP-responsive element modulator and transthyretin (TTR). CD59 is down-regulated in lung cancer urine, which is functionally associated with natural cytotoxicity receptors and activates human NK cell-mediated cytotoxicity [23]. It has been reported that losing CD59 expression in breast tumors correlates with poor survival and may offer a selective advantage for breast cancers, resulting in more aggressive tumors and conferring a poor prognosis for patients [24]. Another protein that showed down-regulation in lung cancer urine was TTR. About 40% of plasma TTR circulates in a tight protein-protein complex with the plasma retinol-binding protein (RBP), which was found to be up-regulated. The formation of a complex with RBP stabilizes the binding of retinol to RBP and decreases the glomerular filtration and renal catabolism of the relatively small RBP molecule [25]. Up-regulated proteins detected are plasma RBP, GM2 activator protein (GM2AP), Ig lambda light chain and Ig kappa chain C regions. Plasma RBP is up-regulated in lung cancer urine, first may be caused of low TTR affected to loss of RBP into urine and the second, RBP concentration is significantly higher in neoplastic tissue may secrete excess RBP from tumor tissue more than the amount of TTR for binding, finally it passes into the urine [26, 27].

Table 2. Protein identification of urinary protein markers in lung cancer urine by MALDI-TOF-MS/MS based on the Swiss-Prot database

Spot no.	Protein name	Acc. No.	Theo. M_r (kDa)/p*I*	App. M_r (kDa)/p*I*	Queries matched	Seq. cov. (%)	Matched sequences
1	CD59 glycoprotein [precursor]	P13987	14.1/6.0	20/4.3	1	9	LRENELTYYCCK
2	Activator of cAMP-responsive element modulator, testis – human	Q8WW21	32.8/8.0	20/4.8	24	73	MTTAHFYCQYCTASLLGKK MTTAHFYCQYCTASLLGKK TTAHFYCQYCTASLLGKK DFCYKVR VRHWHEGCFK HWHEGCFKCTK CTKCNHSLVEKPFAAK DERLLCTECYSNECSSK LLCTECYSNECSSK MEFKGNYWHETCFVCENCR QPIGTKPLISKESGNYCVPCFEK ESGNYCVPCFEKEFAHYCNFCK EFAHYCNFCKK VITSGGTTFCDQLWHK KDLCEEQFMSR CVACSKPISGLTGAK FICFQDSQWHSECFNCGR FICFQDSQWHSECFNCGR FICFQDSQWHSECFNCGRCSVSL VGK CSVSLVGKGFLTQNK GFLTQNKEIFCQK EIFCQK EIFCQKCSSGMDTDI CSSGMDTDI
3	Transthyretin (prealbumin, amyloidosis type I)	P02766	15.9/5.5	18/5.3	1	9	GSPAINVAVHVFRK
4	Plasma retinol-binding protein [Precursor]	P02753	23.0/5.8	25/5.2	2	19	GNDDHWIVDTDYDTYAVQYSCR LLNLDGTCADSYSFVFSR
5	G(M2) activator protein	P17900	20.8/5.2	23/4.3	1	10	SEFVVPDLELPSWLTTGNYR
6	Ig lambda light chain	P01842	11.2/6.8	31/5.8	2	32	AAPSVTLFPPSSEELQANK YAASSYLSLTPEQWK
7	Ig kappa chain C region	P01834	11.6/5.6	16/7.2	2	32	TVAAPSVFIFPPSDEQLK SGTASVVCLLNNFYPR
8	Ig kappa chain C region	P01834	11.0/5.6	16/6.9	1	16	SGTASVVCLLNNFYPR
9	Ig kappa chain C region	P01834	11.6/5.6	15/6.2	2	32	TVAAPSVFIFPPSDEQLK SGTASVVCLLNNFYPR

GM2AP was up-regulated and showed high expression in lung cancer urine. It is an essential cofactor for the degradation of ganglioside GM2 to GM3 by lysosomal β-hexminidase A and is also related to the changing level of ganglioside. Tumor-associated gangliosides may play a role in cancer progression [21, 28]. Tumor cells are synthesized and shed gangliosides into their microenvironments leading to elevated levels of tumor-associated gangliosides in the serum [29, 30]. Changes in the amounts of gangliosides in serum can influence the rate of tumor growth through an undetermined mechanism [31]. High expression of Ig light chains was also found in lung cancer urine samples. There are some reports that Ig light chain may protect against a diseased state; plasma cells are capable of presenting antigenic determinants derived from a secreted monoclonal light chain in an MHC class I context, and of predominantly inducing a monoclonal Ig-specific T-cell response which can contribute to tumor rejection [32]. Ig free light chains can also be transfered immediately to hypersensitivity-like responses in mice and may contribute to the mechanism by which mast cells

Table 3. Protein and gene identification, spot quantity ratio/regulation and description of urinary protein markers in lung cancer urines

Spot no.	Protein name	Gene name	Quantity ratio (normal/lung)/ Regulation	Description
1	CD59 glycoprotein precursor (Membrane attack complex inhibition factor) (MACIF), **CD59_HUMAN**	**CD59**	4.60/Down	Small glycoproteins found on both hematopoietic and nonhematopoietic cells. CD59 restricts the cytolytic activity of homologous complement by binding to C8 and C9 and blocking the assembly of the membrane attack complex. [17, 18].
2	Activator of cAMP-responsive element modulator, testis – human	none	5.06/Down	CREM modulates the transcription of several genes containing a cAMP responsive element motif in their promoter region. Alternative splicing of the CREM gene generates both activator and repressor isoforms that are expressed in a tissue and cell specific manner [19, 20].
3	Transthyretin (prealbumin, amyloidosis type I), **TTHY_HUMAN**	**TTR or PALB**	6.30/Down	A homotetrameric thyroid-hormone transporting protein [21]. About 40% of plasma transthyretin circulates in a tight protein-protein complex with the plasma retinol-binding protein (RBP). Defects in TTR are a cause of amyloidosis. Distinct forms of amyloidosis have been related to different point mutations.
4	Plasma retinol-binding protein [Precursor], **RETB_HUMAN**	**RBP4**	0.06/Up	Delivers retinol from the liver stores to the peripheral tissues. In plasma, the RBP-retinol complex interacts with transthyretin which prevents its loss by filtration through the kidney glomerulus's.
5	G(M2) activator protein, **SAP3_HUMAN**	**GM2A**	0.07/Up	The GM2 activator protein is a small monomeric protein containing a single site for Asn-linked glycosylation. Its only proven *in vivo* function is to act as a substrate specific cofactor for the hydrolysis of GM2 ganglioside by lysosomal beta-hexosaminidase A [22].
6	Ig lambda light chain, **LAC_HUMAN**	**IGLC1**	0.74/Up	High levels of monoclonal IgG (less commonly IgA, rarely IgD or IgE) in the serum and free light chains of the same kappa or lambda type in the urine (Bence-Jones proteinuria) is a hallmark of myeloma disease. Overall, about 70% of myeloma patients have both serum monoclonal Ig and urinary Ig light chains, and the remaining patients have urinary Ig light chains alone without serum monoclonal Ig.
7	Ig kappa chain C region, **KAC_HUMAN**	**IGKC**	0/Up	
8	Ig kappa chain C region, **KAC_HUMAN**	**IGKC**	0/Up	
9	Ig kappa chain C region, **KAC_HUMAN**	**IGKC**	0/Up	

The spot quantity ratio is defined as up- and down-regulation of expressed protein depending on protein spot intensity.

regulate immune diseases [33]. Light chain gene expression, neoplastic and autoimmune disease are being studied to understand the pathways. These identified proteins may become more interesting for diagnostic purposes if their up-regulation can be confirmed by immunohistochemistry or other specific techniques in further analysis.

We examined preparation methods for urine samples from pooled normal and lung cancer patients by proteomic analysis and found the best preparation method for urine samples. According to the results, we chose the sequential preparation of urine samples by gel filtration and ultrafiltration as the selective preparation method to allow preliminarily investigation of urinary protein markers in lung cancer disease. Nevertheless, there are many points that must be considered in the investigation of urinary biomarkers. One is the number of patients and healthy donors available for pooling urine samples. We used five lung cancer patients diagnosed with the same specific cell type and stage for preliminary examination and found the proper sample preparation method. Since proteins in human urine can be affected by many factors such as age, gender, chemotherapy treatment, nutrition and metastasis of malignant tissues, the accuracy of biomarker identification must be concerned with using an adequate sample size of cancer patients to account for these effects. When using samples from large numbers of cancer patients and healthy controls, pooling urine sam-

ples is a suitable way for analysis which can reduce time, cost and allows quick interpretation of data. However, these classified criteria need time and man power for collecting and preparing urine samples. The sample preparation of urine samples, protein patterns, protein identifications and identification of differentially expressed proteins are very important and useful for further studies on the proteome of lung cancer urines. The resulting information is potentially useful for pharmaceutical applications, for example, the preparation of a protein chip of lung cancer urines for clinical diagnosis.

4 Concluding remarks

In this study, we successfully pooled examined preparation methods of pooled normal and lung cancer urines for analysis of urinary proteins and identified some preliminary differentially expressed protein markers. Although the results of HPLC and SDS-PAGE showed few differences in protein patterns between normal and lung cancer urines prepared by the sample preparation, methods compared 2-DE results gave more useful information of different protein patterns related to sample preparations and urine sources between normal and lung cancer patients. We found that the sequential preparation method of urine samples by gel filtration and ultrafiltration provided the largest protein quantity in normal and lung cancer urines, compared to precipitation methods. By proteomic analysis, we compared the protein patterns between normal and lung cancer urines, and found some interesting proteins such as CD59 glycoprotein, TTR, GM2AP and Ig-free light chain to be differentially expressed. These urinary biomarkers may be useful as lung cancer markers, which are needed for further preclinical diagnostic and therapeutically applications.

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