JOURNAL OF PROTEOMICS 85 (2013) 28–43

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Identification of potential bladder cancer markers in urine by abundant-protein depletion coupled with quantitative proteomics

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ARTICLE INFO

Article history:
Received 3 December 2012
Accepted 17 April 2013
Available online 28 April 2013

Keywords:
Urine proteome
Bladder cancer
Biomarkers
Abundant protein depletion

ABSTRACT

In this study, we evaluated the reproducibility of abundant urine protein depletion by hexapeptide-based library beads and an antibody-based affinity column using the iTRAQ technique. The antibody-based affinity-depletion approach, which proved superior, was then applied in conjunction with iTRAQ to discover proteins that were differentially expressed between pooled urine samples from hernia and bladder cancer patients. Several proteins, including seven apolipoproteins, TIM, SAA4, and proEGF were further verified in 111 to 203 individual urine samples from patients with hernia, bladder cancer, or kidney cancer. Six apolipoproteins (APOA1, APOA2, APOB, APOC2, APOC3, and APOE) were able to differentiate bladder cancer from hernia. SAA4 was significantly increased in bladder cancer subgroups, whereas ProEGF was significantly decreased in bladder cancer subgroups. Additionally, the combination of SAA4 and ProEGF exhibited higher diagnostic capacity (AUC = 0.80 and $p < 0.001$) in discriminating bladder cancer from hernia than either marker alone. Using MetaCore software to interpret global changes of the urine proteome caused by bladder cancer, we found that the most notable alterations were in immune-response/alternative complement and blood-coagulation pathways. This study confirmed the clinical significance of the urine proteome in the development of non-invasive biomarkers for the detection of bladder cancer.

Abbreviations: APO, apolipoprotein; LgEs, low grade with early stage; HgEs, high grade with early stage; HgAs, high grade with advanced stage; iTRAQ, isobaric tags for relative and absolute quantitation; LC–MS/MS, liquid chromatography tandem mass spectrometry; proEGF, pro-epidermal growth factor; SAA4, serum amyloid A-4; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TPP, Trans-Proteomic Pipeline; TIM, triosephosphate isomerase.

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http://dx.doi.org/10.1016/j.jprot.2013.04.024

Biological significance
In this study, we evaluated the reproducibility of abundant urine protein depletion by hexapeptide-based library beads and an antibody-based affinity column using the iTRAQ technique. The antibody-based affinity-depletion approach, which proved superior, was then applied in conjunction with iTRAQ to discover proteins that were differentially expressed between pooled urine samples from hernia and bladder cancer patients. Several proteins, including seven apolipoproteins, TIM, SAA4, and proEGF were further verified in 111 to 203 individual urine samples from patients with hernia, bladder cancer, or kidney cancer. SAA4 was significantly increased in bladder cancer subgroups, whereas ProEGF was significantly decreased in bladder cancer subgroups. Additionally, the combination of SAA4 and ProEGF exhibited higher diagnostic capacity in discriminating bladder cancer from hernia than either marker alone. A marker panel composed by two novel biomarker candidates, SAA4 and proEGF, was first discovered and verified successfully using Western blotting. To the best of our knowledge, the associations of urinary SAA4 and proEGF with bladder tumor and kidney cancer have not been mentioned before. In the present study, we discovered and verified SAA4 and proEGF as potential bladder cancer biomarker for the first time.

1. Introduction

Bladder cancer is one of the most common urinary tract carcinomas. According to the most recent estimates of the American Cancer Society, there were 69,250 new cases of bladder cancer in the United States and 14,990 deaths from bladder cancer annually in 2011 [1]. Major risk factors for the development of bladder cancer include cigarette smoking and exposure to specific carcinogens [2].

Prominent among the major clinical problems associated with bladder cancer is the high recurrence rate of superficial tumors; 50–70% of newly diagnosed tumors recur within five years and progression to invasive disease will occur in 10–20% of patients. Therefore, patients with bladder tumors must undergo life-long surveillance [3]. This requirement for lifetime surveillance, taken together with the cost to treat recurrent bladder cancer tumors and complications associated with treatment, imposes a significant economic burden. Screening of high-risk populations may be beneficial in the detection of early-stage tumors before they become invasive or in surveillance to detect recurrence [4]. Thus, the most important clinical challenges are early detection of bladder tumors and discovery of non-invasive stage/grade discriminators or predictors of tumor progression. Integration of multiple markers has the potential to further improve bladder cancer diagnosis and progression monitoring.

Urine, derived from plasma by filtration through the renal glomerulus, has become one of the most widely used clinical samples for biomarker discovery. It is estimated that approximately 70% of urinary proteins are derived from the kidney and urinary tract [5–7]. Many abundant plasma proteins are also present at high concentrations in urine specimens [8–10], making it more difficult to detect potential biomarkers present at low concentrations. Antibody-based affinity-depletion approaches are able to simultaneously remove several of the most abundant proteins from various kinds of body fluids in a single step [11–13]. Simply removing 2 to 20 of the most abundant proteins using immune-depletion approaches has been shown to allow more protein spots to be visualized by two-dimensional electrophoresis or a larger number of proteins to be identified using liquid chromatography (LC)-based proteomic strategies [14,15].

An alternative strategy for enriching low-abundance proteins uses a combinatorial hexapeptide ligand library immobilized on a solid-phase matrix [16–18]. The amount of each specific protein captured by the library is limited by the number of the ligands with suitable affinity interaction. Saturation of affinity ligands limits the amounts of abundant proteins captured, preventing retention of an excess of highly abundant proteins. Therefore, the concentrations of low-abundant proteins are enriched relative to the original composition of the specimen, decreasing the dynamic range of the concentration difference in a biological sample. However, the interaction of a complicated peptide mixture with a random peptide library is an intricate process that can be affected by competition among multiple association/dissociation mechanisms, kinetics of binding affinity, and stoichiometry. When this approach is applied to the discovery of biomarkers in biological fluids, poor reproducibility of the depletion step may cause problems in candidate selection in subsequent proteomic studies. Although hexapeptide-based library beads and columns containing immobilized antibodies are widely used techniques for depletion of abundant proteins in body fluids [16,17], detailed characterizations and comparisons of the two approaches have been limited [17,19], particularly for specimens of urine, a biofluid with low protein levels but a high salt concentration.

In this study, we used the iTRAQ (isobaric tags for relative and absolute quantitation) technique to evaluate the reproducibility of abundant-protein depletion using hexapeptide-based library beads and an antibody-based affinity column—the Agilent human 14 multiple affinity removal system (MARS) column. The antibody-based affinity-depletion approach was then applied in conjunction with iTRAQ to discover proteins differentially expressed between pooled urine samples from age-matched hernia and three subgroups of bladder cancer patients. Several proteins present at markedly different levels were further verified in individual samples using Western blot analyses and a multiplexed Bio-plex assay. The verified proteins were found to be potential markers for early detection and diagnosis of bladder cancer.

2. Experimental procedures

2.1. Clinical specimens

Clinical specimens were collected using a previously described protocol [20]. Briefly, first morning urine samples were collected in the presence of a protease inhibitor cocktail tablet (one tablet per 50 mL urine; Roche, Mannheim, Germany) and sodium azide (1 mM) from hernia volunteers and bladder cancer patients. Hernia patients were served as controls from cancer patients with comparable age and exactly the same procedures of urine sample collection in the first morning after admission before surgical intervention. The collected urine samples were centrifuged at 5000 ×*g* for 30 min at 4 °C within 5 h of collection to remove cells and debris, and the clarified supernatants were stored at −80 °C for further processing. All urine samples were collected at Chang Gung Memorial Hospital, Taoyuan, Taiwan. The study protocol was approved by the Medical Ethics and Human Clinical Trial Committee at Chang Gung Memorial Hospital. The bladder cancer patients recruited in this study were a mixed population containing non-muscle invasive and muscle invasive patients, however, have categorized clearly into three subgroups according to the TNM (tumor, node, metastasis) staging system [21,22]. The three subgroups were low grade with early stage (LgEs), high grade with early stage (HgEs), and high grade with advanced stage (HgAs). LgEs and HgEs BC patients were non-muscle invasive bladder cancer subgroups. HgAs BC patients were muscle-invasive subgroup.

2.2. Concentration and desalting of urine samples

Urine proteins were enriched using a 10-kDa centrifugal filter as described by the manufacturer (Millipore, Carrigtwohill, Ireland). Briefly, 12.5-mL urine samples were centrifuged at 5000 ×*g* for 30 min at 4 °C in the filter tube, and then the tube was refilled with 12.5 mL of 20% acetonitrile/H_2O and centrifuged again. This process was repeated once using pure water for desalting purposes. For iTRAQ labeling, samples were subjected to an additional desalting step using 4 mL H_2O to prevent against possible interference by metabolites in the labeling reaction. The amount of protein in each concentrated/desalted urine sample was measured using a DC protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA), then the sample was lyophilized and stored at −80 °C for subsequent processing.

2.3. Depletion of abundant proteins using MARS column

Urine protein for the discovery phase was obtained from individual urine samples subjected to depletion of 14 abundant proteins using an Agilent Human 14 MARS Column (Agilent, Palo Alto, CA, USA), which contains antibodies raised against human epitopes of albumin, IgG, antitrypsin, IgA, transferrin, haptoglobin, fibrinogen, α2-macroglobulin, α1-acid glycoprotein, IgM, apolipoprotein Al, apolipoprotein All, complement C3, and transthyretin. Urine protein (1 mg) from each individual was processed according to the manufacturer's protocol (Agilent). Briefly, desalted/concentrated urine protein was prepared at 1 mg/40 μL in H_2O, and then diluted to 1 mg/160 μL by the addition of 120 μL buffer A of the MARS column system. The diluted urine protein was then processed using the recommended column run cycle consisting of sample loading, flow-through collection (depleted fraction), washing, and elution of bound proteins by buffer B of the MARS column system. The column was then re-equilibrated for the next depletion run. Depleted and retained urine proteins were concentrated using 3000-MW cut-off concentrators (Millipore, Ireland), quantified using a DC protein assay kit (Bio-Rad Laboratories) and then stored at −20 °C for further use. Abundant-protein-depletion reproducibility and effect on protein identification were evaluated using pooled urine proteins from 12 normal individuals (six males and six females) as a model sample. The model sample was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and LC-tandem mass spectrometry (MS/MS) analyses of 20 fractions generated using basic reverse-phase chromatography.

2.4. Depletion of abundant proteins using ProSpectrum Library hexapeptide beads

ProSpectrum Library PSL-2 resin (4 mg; Proloas, Florence, KY, USA) was loaded onto a spin column (Micro Bio-Spin chromatography columns; BioRad Laboratories) and activated in swelling reagent (50% methanol) for 10 min. After removal of the supernatant, the resin was washed twice with 500 μL of H_2O and then suspended in 500 μL of H_2O. Model sample urine protein (10 mg) was incubated with resin at room temperature for 1 h. After incubation, samples were spun to pellet resin beads. The flow-through fraction containing high-abundance proteins was collected and stored at −20 °C until subsequent SDS-PAGE characterization. Resin beads were washed three times with H_2O. Finally, resin beads were incubated with 500 μL of 9 M urea containing 2% CHAPS for 1 h at room temperature and then spun to elute bound proteins (eluate fraction). The eluate fraction buffer was then changed to water using a centrifugal filter device (Amicon Ultra-4 Centrifugal Filter Device-3000NMWL; Millipore, Cork, Ireland) and stored at −20 °C until subsequent SDS-PAGE characterization and iTRAQ-labeling.

2.5. iTRAQ labeling

The technical performance of the two depletion methods—MARS column and ProSpectrum Library—was evaluated by individually processing the model sample in triplicate using the two depletion methods. An internal standard sample was prepared by mixing equal amounts of the MARS- and ProSpectrum Library-depleted samples. The process-triplicate samples were labeled with iTRAQ reagents using tags 115–117 and mixed with the internal standard (tag 114) for two rounds of iTRAQ experiments, as described in Supplemental Fig. 1. The intensity of the reporter ion was then used to calculate the ratio of the corresponding peptides.

In the biomarker discovery-phase experiment, age-matched hernia patients were chosen as the control subgroup. All hernia and bladder cancer patients were older than 45 years of age. Detailed information about these samples is presented in Supplemental Table 1. The four subgroups in four-plex iTRAQ were classified as hernia and three bladder cancer subtypes, LgEs, HgEs, and HgAs. After abundant-protein depletion, equal

amounts of depleted proteins from patients with the same histological grades or pathological stages were pooled into a subgroup to minimize individual variation (hernia, n = 7; LgEs, n = 4; HgEs, n = 5; HgAs, n = 4) in the discovery-phase experiment. Pooled urine protein (100 μg) from each subgroup was processed according to the manufacturer's protocol for the four-plex iTRAQ (Applied Biosystems, Foster City, CA, USA). Briefly, one unit of iTRAQ reagent (defined as the amount of reagent required to label 100 μg of protein) was thawed and reconstituted in 70 μL of ethanol. Pooled urine protein in each subgroup was reduced, cysteine-blocked, and digested with trypsin. Tryptic peptides of hernia, LgEs, HgEs, and HgAs bladder cancer subgroups were labeled with iTRAQ tags 114, 115, 116 and 117, respectively, by incubation at room temperature for 1 h. The peptide mixtures were then pooled and dried by vacuum centrifugation.

2.6. Fractionation by basic reverse-phase chromatography

For reverse-phase chromatography under basic conditions, tryptic peptides and iTRAQ-labeled peptides were reconstituted in 0.5 mL of buffer C (ammonium hydroxide aqueous solution, pH 10) and loaded onto a 4.6 mm × 150 mm Gemini C18 column containing 3-μm particles with a 160-μm pore size (HPLC Phenomenex, Torrance, CA, USA). The peptides were eluted at a flow rate of 400 μL/min with the following gradient: 2% buffer D (ammonium hydroxide in 100% acetonitrile, pH 10) for 5 min, 2–35% buffer D for 40 min, 35–60% buffer D for 5 min, 60–95% buffer D for 3 min, and 95% buffer D for 3 min. The column was then equilibrated in 2% buffer D prior to the next injection. The elution was monitored by measuring absorbance at 220 nm, and fractions were collected every 1 min. The eluted fractions were readied for nano ESI-LC–MS/MS analysis by pooling into 42 fractions and vacuum drying.

2.7. LC-ESI MS/MS analysis by LTQ-Orbitrap

Each peptide fraction was reconstituted in buffer E (0.1% formic acid in H_2O). Peptides (2 μg) from each fraction were loaded onto a trap column (Zorbax 300SB-C18, 0.3 × 5 mm; Agilent Technologies, Wilmington, DE, USA) at a flow rate of 20 μL/min in buffer E, and separated on a resolving 10-cm analytical BioBasic C_{18} PicoFrit column (inner diameter, 75 μm) with a 15-μm tip (New Objective, Woburn, MA, USA). Peptides were eluted at a flow rate of 0.25 μL/min across the analytical column with linear gradients of 5%–30% buffer F (0.1% formic acid in 99.9% acetonitrile) for 40 min, 30%–45% buffer F for 5 min, and 45%–95% buffer F for 2 min, followed by maintenance in 95% buffer F for 4 min.

The LC setup was coupled in-line to a linear ion trap (LTQ)-Orbitrap (Thermo Fisher, San Jose, CA, USA) operated using XCalibur 2.0 software (Thermo Fisher). Intact peptides were detected in the Orbitrap at a resolution of 30,000. Internal calibration was performed using the ion signal of $(Si(CH_3)_2O)_6H^+$ at m/z 445.120025 as a lock mass [23]. In the analysis of iTRAQ-labeled peptides, peptides selected for MS/MS were fragmented using pulsed-Q dissociation (PQD) and the resulting ion fragments were detected in the LTQ. A data-dependent procedure that alternated between one MS scan followed by three MS/MS scans was applied for the three most abundant precursor ions in the MS survey scan. The m/z values selected for MS/MS were dynamically excluded for 180 s. The electrospray voltage applied was 1.8 kV. Both MS and MS/MS spectra were acquired using the 4 microscan with maximum fill times of 1000 and 100 ms for MS and MS/MS analysis, respectively. Automatic gain control was used to prevent over-filling of the ion trap; 5×10^4 ions were accumulated in the ion trap for generation of PQD spectra. For MS scans, the m/z scan range was 350 to 2000 Da.

2.8. Protein identification and quantitation by sequence database searching

The resulting MS/MS spectra were searched against the European Bioinformatics Institute (http://www.ebi.ac.uk/) non-redundant International Protein Index human sequence database (v3.55 February 2009) containing 75,554 sequences using the MASCOT engine (version 2.2.04; Matrix Science, London, UK) with the Mascot Daemon program (version 2.2.0; Matrix Science). For protein identification, a mass tolerance of 10 ppm was permitted for intact peptide masses and 0.5 Da for PQD-fragmented ions, with allowance for two missed cleavages in the trypsin digests and oxidized methionine as a potential variable modification. iTRAQ (N terminal), iTRAQ (K), and methyl methanethiosulfonate (C) were set as fixed modifications for iTRAQ-labeled samples. The charge states of peptides were set to +2 and +3.

Protein identification for evaluation of depletion approaches by LC-PQD-MS/MS was summarized using Scaffold (version 2_02_01; Proteome Software, Proteome Software Inc., Portland, OR, USA). Identification and quantification of proteins in iTRAQ experiments were validated using the open source Trans-Proteomic Pipeline (TPP) software (Version 4.0). The MASCOT search resulted in a DAT file for each fraction. The MS raw data and DAT files containing peak list information for identified peptides were then processed and analyzed using the TPP software. The TPP software includes PeptideProphet, a peptide probability score program that aids in the assignment of peptide MS spectra [24], and ProteinProphet, a program that assigns and groups peptides to a unique protein or a protein family if the peptide is shared among several isoforms [25]. ProteinProphet allows filtering of large-scale data sets with assessment of prediction sensitivity and false-positive identification error rates. In this study, we used PeptideProphet and ProteinProphet probability scores ≥0.95 to ensure an overall false-positive rate less than 0.53%. The ratio of each protein was quantified using the Libra program, a module within the TPP software package that performs quantification on MS/MS spectra that have multiplexed labeled peptides. The minimum intensity threshold of a reporter ion was 20 in the spectrum of a Libra peptide. The default parameters of the Libra program were used to remove the outlier ratios of peptide quantitation. Each quantified protein contained at least one Libra peptide. Information about the PeptideProphet, ProteinProphet, and Libra programs in the TPP software can be obtained from the Seattle Proteome Center at the Institute for Systems Biology (http://www.proteomecenter.org/).

2.9. Western blot analysis

Urine protein (100 μg) from individual or pooled samples was resolved on an SDS gel and transferred electrophoretically to a

PVDF (polyvinylidene difluoride) membrane (Bio-Rad Laboratories). The membrane was blocked with 5% non-fat dried milk in TBST (Tris-buffered saline [Bio-Rad Laboratories] containing 0.1% Tween-20 [Sigma, St. Louis, MO, USA]) for 1 h at room temperature. The following antibodies were used for Western blot analysis: anti-apolipoprotein A4 (anti-APOA4, 1:10,000, LS-B2417; Lifespan Biosciences, Seattle, WA, USA), anti-vitamin D-binding protein (anti-VDBP, 1:2000, sc-32899; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-triosephosphate isomerase (anti-TIM, 1:1000, sc-30145; Santa Cruz Biotechnology), anti-serum amyloid A-4 protein precursor (anti-SAA4, 1:1000, H00006291-D01P; Abnova), and anti-EGF (anti-epidermal growth factor, 1:2000, sc-275; Santa Cruz Biotechnology). The membranes were probed by incubating first with primary antibody and then with horseradish peroxidase-conjugated secondary antibody, and developed using enhanced chemiluminescence detection according to the manufacturer's instructions (PerkinElmer, Netherlands or Millipore, MA, USA). An internal standard from a pooled urine sample was included in each Western blot analysis and used to normalize the intensity of each target protein detected.

2.10. *Immunobead-based suspension array system for detection of urine apolipoproteins*

The urine levels of six apolipoproteins (APO A1, A2, B, C2, C3, and E) were determined with the MILLIPLEX MAP Human Apolipoprotein Panel kit (Millipore, MA, USA) using the Bio-Plex system (Bio-Rad Laboratories). The assay procedure was a modification of the blood sample-suitable protocol provided by Millipore. Briefly, filter-bottom, 96-well microplates were pre-wetted with wash buffer (Millipore) and blocked for 10 min with assay buffer (Millipore). A standard curve was generated by preparing 5-fold serial dilutions of appropriate standards in assay buffer. Ten microliters of prepared standards and urine samples (2-fold dilution) were added into wells containing 90 μL of assay buffer with immunobead mixtures. Microplates were then incubated for 50 min at room temperature on a microtiter shaker in the dark and then washed three times with wash buffer using a vacuum manifold (Millipore). The mixture of biotin-conjugated detection antibodies was added to the wells and plates were incubated for 30 min. After washing, phycoerythrin–streptavidin was added and plates were incubated for 30 min. Finally, the washed immunobeads were resuspended in sheath buffer (Bio-Rad Laboratories) and analyzed using the Bio-Plex 200 system (Bio-Rad Laboratories). Standard curves and analyte concentrations were obtained using Bio-Plex Manager software version 4.2 (Bio-Rad Laboratories).

2.11. *Statistical analysis*

The statistical package SPSS 13.0 (SPSS Inc., Chicago, IL, USA) was used for all Western blotting and Bio-plex systems analyses of protein expression levels in individual samples. Differences in concentration levels of targeted urine proteins in relation to different clinical parameters were analyzed using the nonparametric Mann–Whitney U test. Receiver operator characteristic (ROC) curve analysis and area under the curve (AUC) analysis were applied to detect the optimal cutoff point that yielded the highest total accuracy with respect to discriminating different clinical classifications. A p-value less than 0.05 was considered statistically significant.

2.12. *Network analysis*

A network analysis of differentially expressed proteins identified in each clinical set was performed using the MetaCore analytical suite version 5.3 (GeneGo, Inc., St. Joseph, MI, USA). Hypothetical networks of proteins from our experiments and proteins from the MetaCore database were built using the algorithms integrated within MetaCore. The statistical significance of the identified networks was based on p-values, which are defined as the probability that a given number of proteins from the input list will match a certain number of gene nodes in the network. The relevant pathway maps were then prioritized based on their statistical significance ($p < 0.001$).

3. Results

3.1. *Improvement of protein identification using two abundant-protein-depletion approaches*

In order to more easily isolate and identify minor proteins in urine, we used the MARS column to chromatographically remove 14 high-abundance proteins from two pooled urine samples. Removal of these abundant proteins is expected to improve the subsequent LC/MS and electrophoretic analysis of the urine sample by effectively expanding the dynamic range of the detection. A second approach, ProSpectrum peptide library, was also used to process urine proteins to compress the protein concentration range. Two pooled clinical urine protein samples, one from nine bladder cancer patients and the other from nine hernia controls, were used to assess the improvement in protein identification after processing samples individually with the two depletion approaches. Each sample was fractionated into 20 fractions using basic reverse-phase chromatography and analyzed using nano LC–MS/MS. The results of this analysis are summarized in Table 1, which shows the numbers of identified peptides and proteins with false-positive rates less than 0.5%. Prior to depletion, a total of 323 proteins (1390 unique peptides) were identified in the pooled hernia patient urine sample, and 309 proteins (1548 unique peptides) were identified in the urine sample from bladder cancer patients. Following depletion of high-abundance proteins using the MARS Hu-14 depletion column, 512 proteins (2698 unique peptides) were identified in the hernia patient sample and 479 proteins (2377 unique peptides) were identified in the bladder cancer sample. After depletion using ProSpectrum Library beads, 495 proteins (2525 unique peptides) were identified in the hernia sample and 551 proteins (3480 unique peptides) were identified in the bladder cancer sample. These results indicate that both the MARS Hu-14 depletion column and ProSpectrum Library beads resulted in an increase in the number of identified proteins (1.53- to 1.78-fold) and peptides (1.54- to 2.25-fold) in the urine proteome, an improvement in the detection of minor proteins that is expected to improve biomarker discovery in the urine proteome.

Table 1 – Protein identification of hernia and bladder cancer urine proteome before and after abundant protein depletion by MARS column and ProSpectrum Libraries™ Hexapeptide Beads.

Description	Without depletion		MARS Hu-14 depletion [a]		Prospectrum libraries depletion [b]	
	Hernia	Bladder cancer	Hernia	Bladder cancer	Hernia	Bladder cancer
Identified proteins	323	309	512	479	495	551
Identified peptides	1390	1548	2698	2377	2525	3480

[a] The total amount of input protein was 1 mg.
[b] The total amount of input protein was 10 mg.

3.2. *Evaluation of the reproducibility of the two abundant-protein-depletion approaches using the iTRAQ platform*

Fig. 1 shows representative results of an SDS-PAGE analysis of urine protein patterns from a pooled normal urine specimen from 12 healthy volunteers (six males and six females) before and after preparation by ProSpectrum Library beads and the MARS Hu-14 depletion column. The SDS-PAGE analysis revealed a dramatic change in the protein profiles of urine samples after depletion. The average recovery rates were 26.5% ± 1.9% and 1.5% ± 0.1% for the MARS column (1 mg starting urine protein) and ProSpectrum Library beads (10 mg starting urine protein), respectively. As shown in Fig. 1, the MARS column removed the specific target proteins of the antibodies; however, many non-target proteins were also removed from the original samples. Depleted fractions from samples processed using ProSpectrum Library beads tended to be enriched for low-molecular-weight proteins (<21 kDa). This observation is consistent with the findings of Bandhakavi et al., who reported that the use of hexapeptide beads enriched proteins with small to medium molecular weights in the saliva proteome [16]. This phenomenon is important for urine proteome analysis because major urine proteins are relatively smaller owing to the filtration function of kidney. Low-abundance proteins with low molecular weight may thus become more easily detectable after processing with hexapeptide beads.

The pooled normal urine sample was then used to evaluate the two approaches in terms of reproducibility of sample preparation. A mixture of depleted protein samples prepared by the two approaches was labeled with the iTRAQ 114 tag and used as an internal standard. Equal amounts of depleted protein samples, labeled with 115, 116 and 117 tags in two iTRAQ experiments, were processed independently in triplicate according to the experimental design shown in Supplemental Fig. 1. A total of 246 proteins were quantified between the two iTRAQ experiments. A detailed list of proteins and peptides obtained through iTRAQ analysis is presented in Supplemental Table 2. To assess the experimental variation that occurred at the depletion step, we calculated the coefficient of variation (CV) of the 246 quantified proteins using 115/114, 116/114, and 117/114 ratios. We further classified the quantified proteins into subgroups according to the number of Libra peptides, defined as peptides used to quantify a specific protein, and calculated the average CV values of the subgroups. Average CV values plotted

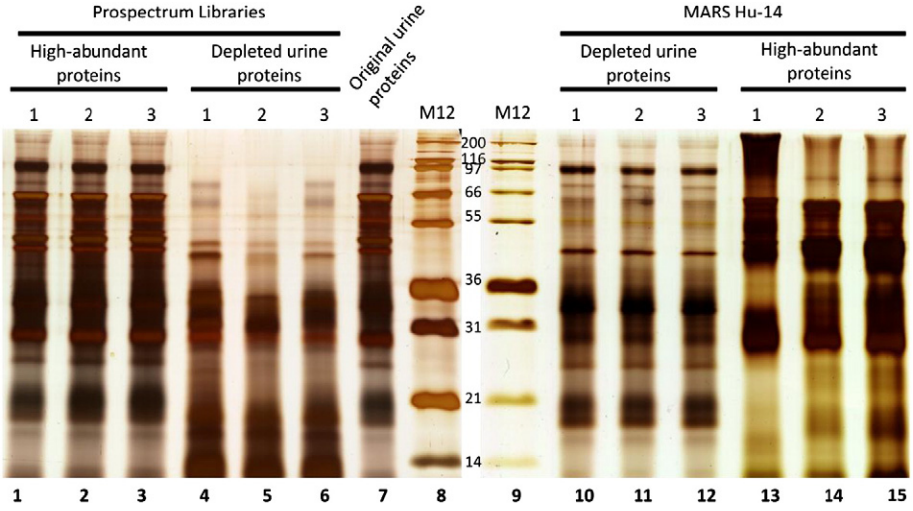

Fig. 1 – Protein profiles of a pooled model sample obtained by processing with ProSpectrum Library beads and a MARS column compared using SDS-PAGE. Equal amounts of protein (4 μg/lane) were separated on 12.5% gels and silver stained. Lanes 1–3 (triplicates), fractions of high-abundance proteins generated using ProSpectrum Library beads; lanes 4–6 (triplicates), fractions of depleted proteins generated using ProSpectrum Library beads; lane 7, original protein without depletion. Lanes 8 & 9, reference molecular weight markers; lanes 10–12 (triplicates), fractions of depleted proteins generated using a MARS Hu-14 column; lanes 13–15 (triplicates), fractions of high-abundance proteins generated using a MARS Hu-14 column.

against the Libra peptide number of a protein is shown in Fig. 2, which indicates that the more Libra peptides used to quantify a protein, the smaller the CV value. A smaller CV should facilitate the selection of biomarker candidates for further verification. The regression analysis shown in Fig. 2 indicates that the average CV values of the two depletion approaches were each less than 25% (Libra peptide number ≥1). When a protein was quantified with one Libra peptide, CV values were ~15% and 20% for MARS and ProSpectrum Library beads, respectively. When four Libra peptides per protein were used, CV values were kept close to 10% for MARS column-depleted samples but were slightly greater than 15% for samples depleted by Spectrum Library beads. CV values less than 10% in ProSpectrum Library bead-depleted samples were only observed for proteins quantified using more than ten Libra peptides, which may only be attainable for medium- to high-abundance proteins. The MARS column thus appears to be more quantitatively reproducible for the depletion of abundant urine proteins under our experiment conditions. Therefore, to discover candidate biomarkers for bladder cancer, we used the MARS column in conjunction with iTRAQ-labeling and LC–MS/MS in subsequent comparative urine proteomic studies.

3.3. *Comparative analysis of the depleted urine proteome in the discovery of bladder cancer biomarkers*

For the discovery phase of this study, equal amounts of abundant-protein-depleted urine protein samples from individuals were pooled into subgroups. The four subgroups in four-plex iTRAQ were classified as hernia and bladder cancer subtypes LgEs, HgEs, and HgAs, determined according to the TNM staging system [21,22]. A total of 644 urine proteins were identified in the iTRAQ experiment, of which 586 had at least one Libra peptide (Supplemental Table 3). iTRAQ ratios of this latter group were quantified, and those displaying a fold-difference ≥2 or ≤0.5 between any tumor subgroup and the hernia subgroup were defined as increased or decreased proteins, respectively, as described in our previous study [20]. We classified these quantified proteins into the following four categories: (I) bladder cancer > hernia: 152 proteins; (II) bladder cancer ≤ hernia: 130 proteins; (III) non-significant difference: 225 proteins; and (IV) others: 79 proteins. The numbers of quantified proteins in each subgroup are summarized in Table 2.

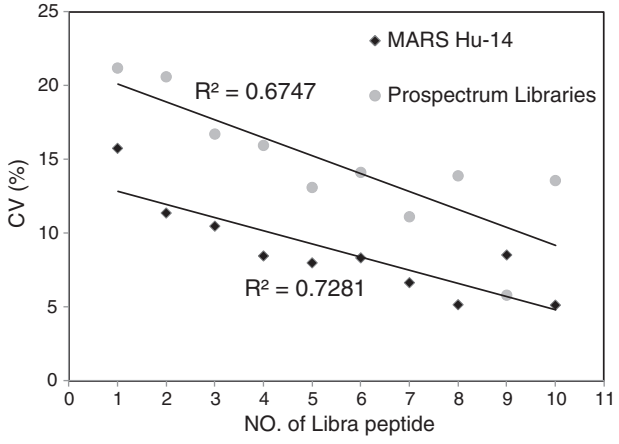

Fig. 2 – Reproducibility of the abundant-protein-depleted urine proteome using each of the two depletion approaches. Reproducibility was evaluated by plotting average CV values versus Libra peptide number of an identified protein.

Table 2 – Protein identification and quantification results from iTRAQ-labeled sample set.

Classification	Definition	Protein number
Identified proteins		644
Quantified proteins (QP)		586
(I) BC[a]>hernia[b]	All Ratios[c] ≥ 1, and Any Ratio[d] ≥ 2	152
(II) Hernia>BC	All Ratios ≤ 1, and Any Ratio ≤ 0.5	130
(III) No significant change	0.5 < All Ratios < 2	225
(IV) Others	(QP)–(I)–(II)–(III)	79

[a] BC: Bladder cancer.
[b] Hernia: Non-tumor control.
[c] All Ratios: 115/114, 116/114 and 117/114 ratios.
[d] Any Ratio: 115/114, 116/114 or 117/114 ratio.

3.4. *Validation of iTRAQ quantitative results in pooled urine samples by Western blot analyses*

To avoid possible interference due to co-elution of isobaric peptides and to confirm the iTRAQ results, we selected three proteins, TIM, VDBP and proEFG, for quantification by Western blot analyses of the pooled samples originally used for the iTRAQ experiment. iTRAQ ratios and Western blot analyses showed similar quantitative trends in proteins between bladder cancer and control subgroups (Fig. 3); the levels of TIM and VDBP were increased and proEGF was decreased in all subgroups of bladder cancer specimens. The Western blotting results confirmed the reliability of MS-based quantitation for the urine proteome.

3.5. *Biomarker verification of apolipoproteins using a Bioplex assay*

In the iTRAQ dataset, apolipoproteins APOA1, APOA2, APOA4, APOB, APOE, and APOM were present at elevated levels in bladder cancer urine specimens. Therefore, we first sought to verify the elevated levels of multiple apolipoproteins in 111 individual urine samples (48 hernia, 20 LgEs, 33 HgEs, and 10 HgAs) using the Human Apolipoprotein Kit, which is commercially available as a 96-well plate immunoassay for multiplexed detection of APOA1, APOA2, APOB, APOC2, APOC3, and APOE. As shown in Fig. 4A–F, Supplemental Table 4 and summarized in Table 3, this assay revealed that these six urine apolipoproteins were significantly higher in BC subgroups compared to hernia (n = 111), validating the results of the iTRAQ analysis. The levels of all six proteins were significantly different between bladder cancer and hernia ($p < 0.001$ and AUC > 0.7); thus, each protein was able to differentiate these conditions. APOA1 had the largest AUC value (0.875). This result re-confirms our previous work on the quantitation of urinary APOA1 in individual hernia and

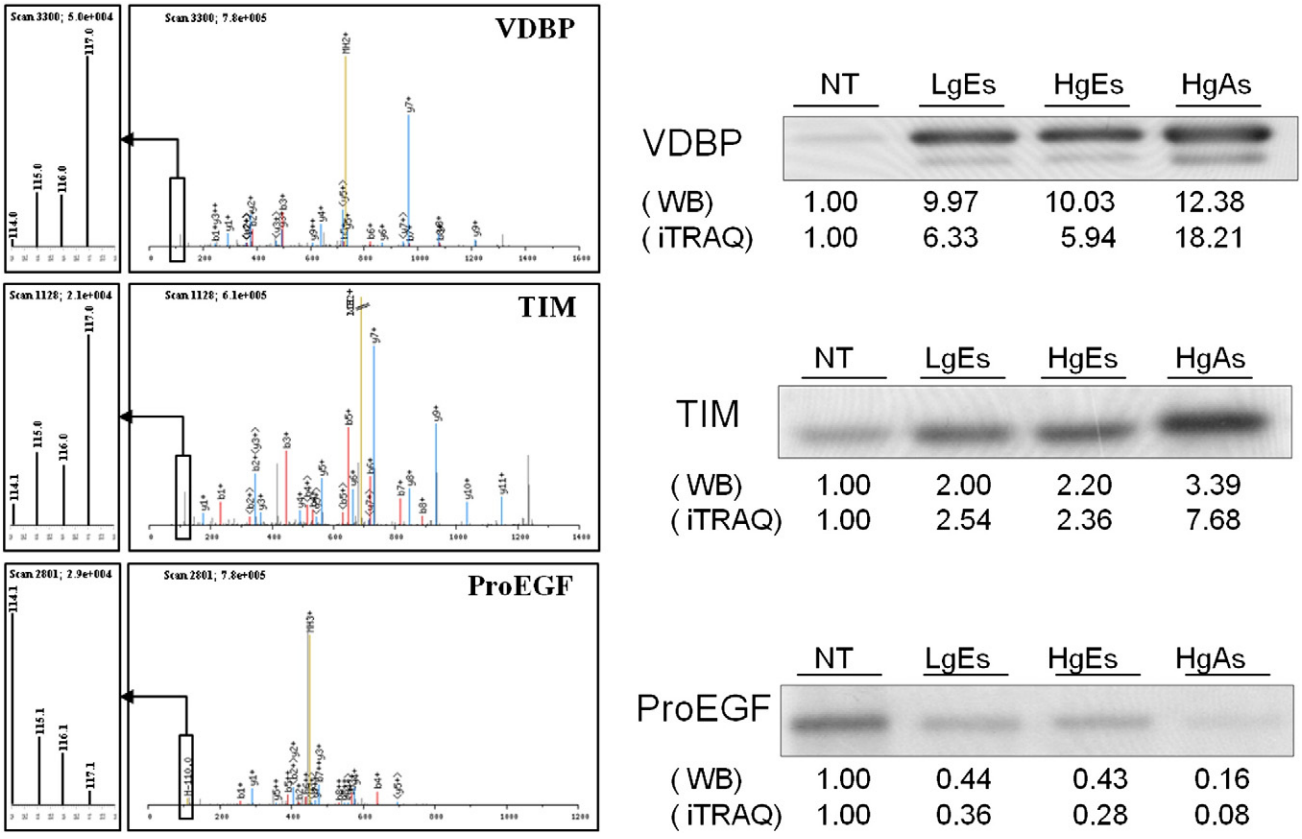

Fig. 3 – Verification of the three selected proteins, VDBP, TIM and proEGF, in pooled urine samples by Western blot analyses. Proteins (50 μg) from pooled urine samples used for the original iTRAQ experiment were subjected to Western blot analysis (WB). Fold-differences of target protein levels in cancer subgroups relative to the hernia group are denoted below each blot (right panel). LC–MS/MS quantification results (in the low mass reporter ion region) and identification of the three selected candidate biomarkers are shown in the left panel.

bladder cancer specimens using APOA1 ELISA and MRM-MS assays [10,20]. AUC values of the remaining five proteins ranged from 0.739 to 0.864. Five of the six validated proteins (APOA1, APOA2, APOB, APOC2 and APOC3) also showed significant differences between hernia and low grade/early stage bladder cancer, with AUC values ranging from 0.713 to 0.791. These findings indicate that the six apolipoproteins are potential urine biomarkers for the diagnosis of bladder cancer. With the exception of APOE, they may also be useful in the early detection of bladder tumors.

3.6. *Verification of potential biomarkers APOA4, TIM, SAA4 and ProEGF by Western blot analysis in 163 to 174 individual urine samples*

On the basis of the iTRAQ results and potential relevance in human cancer, we selected four additional candidates, APOA4, TIM, SAA4 and ProEGF, for verification in individual samples by Western blotting. According to the iTRAQ results, urinary concentrations of APOA4, TIM, and SAA4 were elevated and those of ProEGF were decreased in all subgroups of pooled bladder cancer. We then analyzed the levels of the four proteins in 163 to 174 individual urine samples by Western blotting; the results are shown in Figs. 5A and 6A, Supplemental Figs. 2A and 3A, and, Supplemental Table 4. The LgEs/hernia, HgEs/hernia, and HgAs/hernia ratios for APOA4 were 1.0, 5.6, 4.4, and 5.8, respectively, in the individual samples (Supplemental Fig. 2B). For TIM, the ratios for LgEs/hernia, HgEs/hernia, and HgAs/hernia were 0.9, 1.1, and 1.6, respectively (Supplemental Fig. 3B). However, a statistical analysis indicated that neither APOA4 nor TIM levels were significantly increased in bladder cancer (Table 4), indicating that TIM and APOA4 are probably not useful urine biomarkers for bladder cancer diagnosis.

A Western blot analysis of the remaining two candidate proteins, SAA4 and ProEGF, using 174 individual samples showed a similar trend to the iTRAQ results. SAA4 levels were increased 4.0- to 17.0-fold in bladder cancer compared to hernia subgroups (n = 174; Fig. 5B), and ProEGF was decreased by 70–90% in bladder cancer compared to hernia subgroups (n = 163; Fig. 6B). Both SAA4 and ProEGF levels were significantly altered in all bladder cancer specimens ($p \leq 0.05$), indicating SAA4 and ProEGF are potentially useful biomarkers for detecting bladder cancer. Table 4 summarizes the diagnostic efficacies of urine SAA4 and ProEGF in bladder cancer detection or as stage/grade discriminators. The AUC values derived from ROC curve analyses were 0.68 and 0.71 for SAA4 and ProEGF, respectively, for discriminating bladder cancer

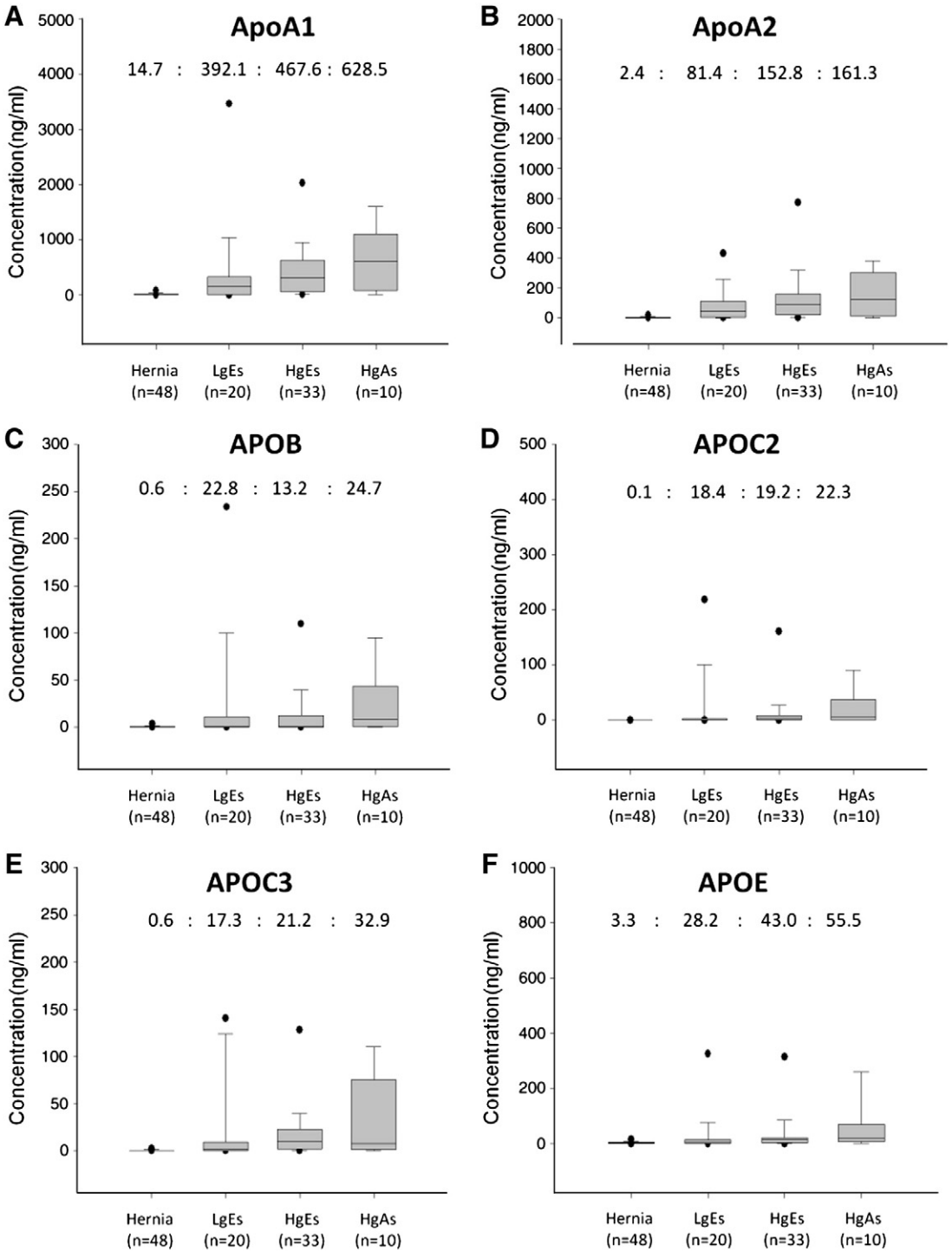

Fig. 4 – Validation of six apolipoproteins using a multiplexed Bio-plex assay. Concentrations of six urine apolipoproteins were elevated in BC subgroups compared to hernia (n = 111). All six proteins were able to differentiate bladder cancer from hernia with statistical significance.

from hernia. Patients with high-grade bladder cancer showed a higher concentration of urinary SAA4 than patients with low-grade bladder cancer (AUC = 0.75 and $p < 0.001$; n = 99). The decreased level of ProEGF was also significantly different between hernia and the LgEs bladder cancer subgroup (n = 88), suggesting promise in the early detection of cancer (Table 4).

It is thought that biomarker panels consisting of multiple biomarkers can provide greater specificity and sensitivity than each biomarker alone, as each of the single biomarker contributes independent diagnostic information [26]. The use of a biomarker panel usually relies on an interpretation of the pattern of the different biomarkers in relation to one another rather than the absolute levels of each biomarker. Accordingly,

Table 3 – Summary of the *p* values, AUC values of the ROC curves, sensitivity and specificity of the six apolipoproteins assessed by Bioplex in constant volume of individual urine samples.

Protein	Hernia vs. LgEs (n = 68)		Hernia vs. all BC (n = 111)		Lg BC vs. Hg BC (n = 63)	
	p value	AUC	*p* value	AUC	*p* value	AUC
ApoA1	**<0.001**	**0.777**	**<0.001**	**0.875**	0.053	0.652
ApoA2	**<0.001**	**0.791**	**<0.001**	**0.864**	0.084	0.636
ApoB	**<0.001**	**0.719**	**<0.001**	**0.739**	0.837	0.516
ApoC2	**<0.001**	**0.738**	**<0.001**	**0.838**	**0.025**	0.676
ApoC3	**<0.001**	**0.713**	**<0.001**	**0.835**	**0.024**	0.677
ApoE	0.11	0.623	**<0.001**	**0.745**	**0.046**	0.657

Statistically significant values (*p* value ≤0.050 or AUC values >0.7) are highlighted in bold.

we evaluated the diagnostic efficacy of a panel combining the two discovered biomarkers, SAA4 and ProEGF. The combination of SAA4 and ProEGF exhibited higher diagnostic capacity (AUC = 0.80 and $p < 0.001$; n = 163) than either marker alone (Table 4).

3.7. *Correlations of SAA4 and ProEGF with clinical data*

In this study, we used age-matched elder hernia patients as normal controls. The average ages of control and cancer subgroups were all more than 60 years old (Supplemental Table 1). In this study, age-matched hernia patients were chosen as the control subgroup because of no pathological problems in their urological system. The possible changes of urine proteome caused by age difference could be excluded. We furthered subgrouped the smoking and non-smoking patients. The detailed information of patient gender and smokers are summarized in Supplemental Table 5A. Although the gender was not completely matched between hernia and cancer groups due to the disease characteristics, the chi square test was performed to confirm that the expression levels of SAA4 alone, proEGF alone, and SAA4 and proEGF panel were not correlated with age, gender, or smoking in both hernia and cancer patients (all *p*-values were not smaller than 0.05, Supplemental Table 5A). Therefore, the changes of the discovered marker (panel) should not be caused by the age, sex, or smoking.

Supplemental Table 5B showed that the discovered marker panel was correlated statistically with tumor size (2 cm as cutoff value) and tumor invasion status ($p < 0.05$), but not correlated with metastasis or distant metastasis. A significantly changes in levels of SAA4 (P = 0.009) and SAA4-ProEGF panel ($p < 0.001$) were observed in T1–T4 tumors as compared with Ta tumors. Taken together, our data suggest that the newly discovered urinary marker panel might have the potential to detect bladder cancer as well as to monitor the possibility of progression after initial resection of different tumor size and tumor grade of high-risk individual.

In clinical scenarios, these urinary tumor markers or marker panel could be clinically applicable in three circumstances (Supplemental Fig. 4). Firstly, in initial approach for any suspicion of bladder urothelial carcinoma, the patient may be much sure of this disease entity in the presence of gross hematuria, especially when the patient is not ready or refuses any invasive procedure in the beginning. Secondly, in the follow-up phase after definite tumor resection is completed, the markers may provide as a surrogate for repeated cystoscopy every 3 to 6 months for several years. An intriguing instance may be for any patient who is high tumor grade or carcinoma in situ that likely to be recurrent and high probability of progression to muscle invasion. High index of suspicion of recurrence may be warranted if these sensitive markers are positive but negative image study and negative cystoscopy. Thirdly, for patient with advanced stage after radical cystectomy, the only urinary marker is urine cytology which is recognized as lack of sensitivity. These convenient urinary markers may prompt attention to prevent progression to incurable metastatic disease.

3.8. *Assessment of the diagnostic efficacy of SAA4 and ProEGF in detecting kidney cancer*

The renal and urological system produces urine and maintains homeostasis. Urine is produced in the kidneys, and then is collected and stored by the bladder before disposal through urination. Bladder tumors account for 90–95% of urothelial carcinomas, the most common malignancy of the urinary tract [27]. Transitional cell carcinoma (TCC) accounts for about 90% of bladder cancer [4]. Although the candidate biomarkers described above were discovered in urine specimens from bladder cancer patients, they may indicate the dysfunction of organs in the urinary tract in addition to the bladder. Thus, these candidate biomarkers should also be validated in urological carcinomas other than bladder cancer to clarify their relevance to individual urinary tract organs and tumor locations. Kidney cancer is more common in men than in women (2:1) and occurs in patients between the ages of 50 and 70 years, similar to bladder cancer. There are several types of kidney cancer; the two most common types are renal cell carcinoma (RCC) [28,29] and urothelial cell carcinoma of the renal pelvis. RCC accounts for more than 90% of kidney carcinomas [30]. Nearly all renal pelvis cancers, which constitute less than 10% of kidney carcinomas [30], are of the TCC type similar to the major type of bladder cancer. Therefore, to further explore the clinical utility of urinary SAA4 and proEGF, we measured their levels in RCC and TCC subtypes of kidney cancer.

Table 5 summarizes the changes in urinary SAA4 and ProEGF levels in kidney cancer specimens and the diagnostic efficacy of SAA4 and ProEGF in detecting kidney cancer. We found that urinary SAA4 levels were significantly elevated in TCC (70.6-fold increase; $p < 0.001$ and AUC = 0.934) and RCC (8.0-fold increase; $p < 0.001$ and AUC = 0.826) subgroups of kidney cancer compared to hernia (Fig. 5B). Average levels of urinary ProEGF were decreased by 70% in TCC-type kidney cancer, but showed no significant difference between RCC-type kidney cancer and hernia (Fig. 6B). Thus, this analysis revealed that the changes in urinary SAA4 and ProEGF levels followed similar trends in both bladder cancer and TCC-type kidney cancer subgroups (Tables 4 and 5). SAA4 levels were increased in urine specimens from bladder cancer, TCC and RCC of kidney cancer with statistic *p*-values, and urinary ProEGF levels was only decreased significantly in bladder cancer, but not in kidney cancer. SAA4 can be used to differentiate bladder cancer from TCC-type kidney cancer ($p < 0.001$ and AUC = 0.831; n = 116), whereas ProEGF levels are capable of differentiating bladder cancer from RCC-type kidney cancer ($p < 0.001$ and AUC = 0.824; n = 123).

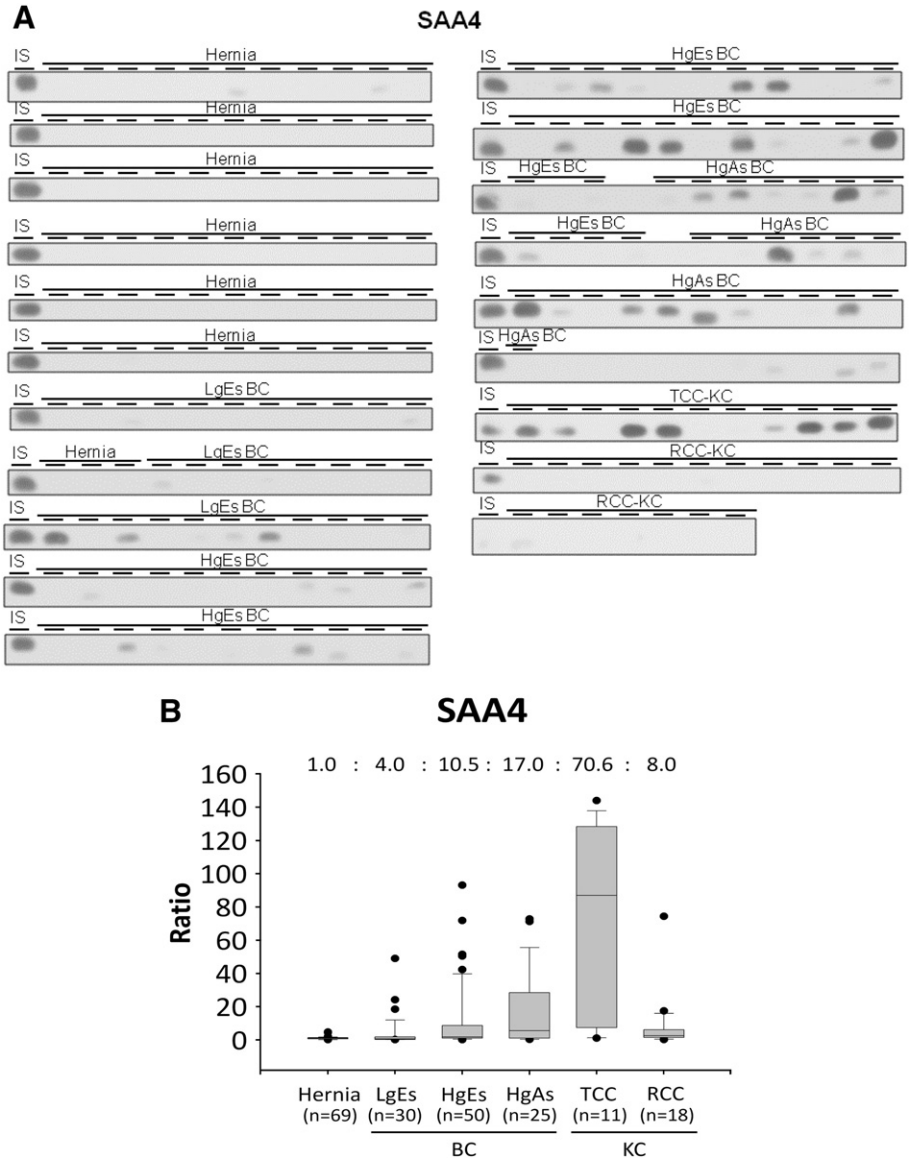

Fig. 5 – Validation of SAA4 in 203 individual urine samples by Western blot analysis. The sample cohort contained 69 hernia controls, 105 samples of bladder cancer (30 LgEs, 50 HsEs, and 25 HgAs subgroups), and 29 samples of kidney cancer (11 TCC and 18 RCC subtypes). (A) Equal amounts of protein prepared from individual urine samples were separated by SDS-PAGE, transferred to PVDF membranes, and probed with antibodies against SAA4. (B) Quantification of the results in A. The average fold-difference between each subgroup and the hernia subgroup is labeled above each box plot. A pooled urine sample prepared from six bladder cancer patients was used as an internal standard to normalize the intensity of each target protein detected. The horizontal lines in each box plot denote the 10th, 25th, 50th, 75th, and 90th percentiles of the data distribution.

Combining SAA4 and ProEGF as a panel creates the potential to differentiate hernia from bladder cancer, low-grade from high-grade bladder cancer, hernia from all kidney cancers, hernia from TCC-type kidney cancer, hernia from RCC-type kidney cancer, RCC- from TCC-type kidney cancer, and bladder cancer from RCC- and TCC-type kidney cancer (Table 5).

3.9. *MetaCore analysis of biological networks associated with urinary proteins dysregulated in bladder cancer*

Finally, in order to interpret global changes in the urine proteome associated with bladder cancer, we analyzed the 361 proteins whose levels were significantly different between hernia controls and bladder cancer patients using MetaCore software. The analysis revealed that 17 pathways were significantly associated with these dysregulated urinary proteins ($p < 0.001$), including immune-response, blood-coagulation, cell-adhesion, development, transport, and cytoskeleton-remodeling pathways (Supplemental Table 6). As shown in Supplemental Fig. 5A–B, the most significant biological networks identified were the immune-response/alternative complement pathway and blood-coagulation pathway, with p-values of 1.66×10^{-21} and 6.99×10^{-20}, respectively.

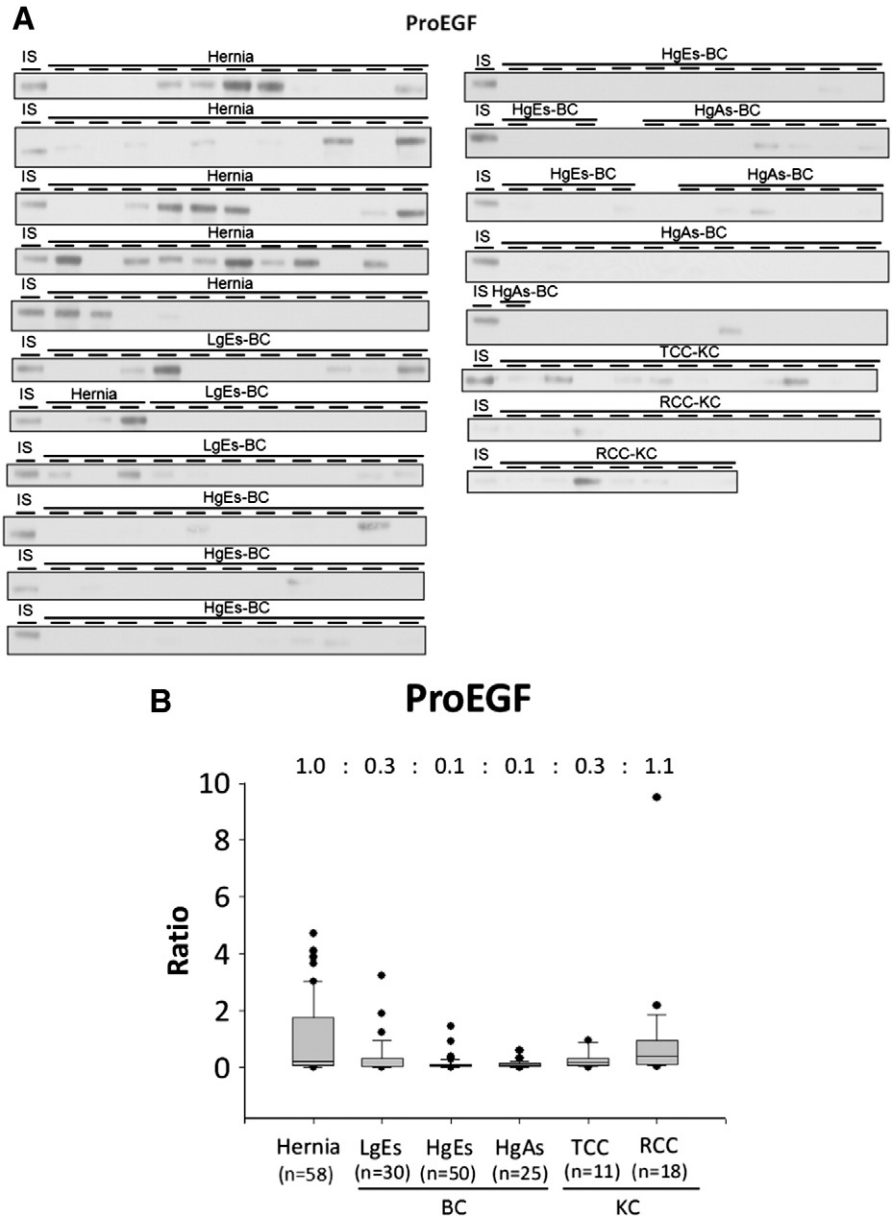

Fig. 6 – Validation of proEGF in 192 individual urine samples by Western blot analysis. The sample cohort used and experimental details were the same as those described in Fig. 5, except anti-proEGF antibody was applied to detect proEGF in urine samples.

4. Discussion

Although there have been some studies for bladder cancer biomarker discovery in urine, those potential markers discovered, such as APOA1 [20,31], myoglobin, and fibrinogen [31], belong to abundant protein category found in a variety of human body fluids including serum/plasma. In this study, we used accurate iTRAQ-based quantitative technology to evaluate the reproducibility of two abundant protein–depletion approaches for urine biomarker discovery and identified proteins that were differentially expressed in the urine proteome of bladder cancer patients. With the incorporation of abundant protein depletion in this work, more medium-minor abundant proteins could be quantified for the first time in urine proteome. Therefore, the result of quantitative analysis of depleted urine proteome provided novel candidates for further investigation as biomarkers for the non-invasive detection of bladder cancer. A marker panel composed by two novel biomarker candidates, SAA4 and proEGF, was first discovered and verified successfully using Western blotting. To the best of our knowledge, the associations of urinary SAA4 and proEGF with bladder tumor and kidney cancer have not been mentioned before. In the present study, we discovered and verified SAA4 and proEGF as potential bladder cancer biomarker for the first time.

The specificity issue represents one of the major challenges in bladder cancer biomarker discovery. This issue is complicated by hematuria—the presence of blood-derived proteins in urine,

Table 4 – Summary of statistical analyses of the efficacy of APOA4, TIM, SAA4 and ProEGF urine levels in detecting bladder and kidney cancers.

Protein name	Hernia vs. all BC		Hernia vs. LgEs		Lg vs. Hg		Es vs. As	
	p-value for diagnosis	AUC	*p*-value for early detection	AUC	*p*-value for grade differentiation	AUC	*p*-value for stage differentiation	AUC
APOA4	0.400 (n = 174)	0.538	0.796 (n = 99)	0.484	0.483 (n = 105)	0.544	0.781 (n = 105)	0.519
TIM	0.686 (n = 174)	0.518	0.223 (n = 99)	0.577	0.164 (n = 105)	0.587	0.109 (n = 105)	0.606
SAA4	**<0.001** (n = 174)	0.684	0.508 (n = 99)	0.542	**<0.001** (n = 105)	**0.749**	**0.008** (n = 105)	0.677
ProEGF	**<0.001** (n = 163)[a]	**0.705**	**0.003** (n = 88)[a]	0.691	0.910 (n = 105)[a]	0.493	0.863 (n = 105)[a]	0.511
Panel (SAA4 + ProEGF)	**<0.001** (n = 163)	**0.796**	**0.033** (n = 88)	0.639	**<0.001** (n = 105)	**0.719**	**0.037** (n = 105)	0.638

Statistically significant values (p value ≤0.050 or AUC values >0.7) are highlighted in bold.
[a] Measured by Western blot analyses based on constant amount of total urine protein.

which is a common symptom in patients with bladder cancer. The urine red blood cells (RBC) test, the most common way to measure the presence of hematuria in urine. We have examined the relationship between hematuria and the urinary levels of SAA4, proEGF and combined panel, which showed a low correlation (r = −0.196–0.453, n = 95–116, Supplemental Fig. 6). Therefore, the alteration of urine levels of SAA4 and proEGF in bladder cancer patients could not be predicted by the degree of hematuria in urine.

4.1. *Biological significance of SAA4 and ProEGF in disease*

Members of the serum amyloid A (SAA) family of acute-phase proteins are expressed at sites of inflammation [32]. SAA4 exists as a minor apolipoprotein on high-density lipoprotein in plasma and is a minor acute-phase reactant in humans [33]. SAA4 can be used as a possible nutritional marker of hepatic protein synthesis in the absence of inflammation [33]. To the best of our knowledge, the alteration of SAA4 levels in body fluids of diseased patients are limited. We found more than a 3-fold increase in urinary SAA4 levels in bladder cancer and kidney cancer specimens. In addition, the degree to which SAA4 levels were increased was related to the disease status of bladder cancer patients, suggesting the potential use of urinary SAA4 levels as a grade discriminator. Taken together, these findings indicate that urinary SAA4 is a novel biomarker for detecting urological system tumors. Additional studies using a larger set of clinical specimens will be required to confirm the clinical utility of urinary SAA4.

We also found here for the first time that urinary proEGF levels were significantly decreased in bladder cancer patients (Fig. 6 and Table 4). Information regarding the association of proEGF with human diseases is also very limited. Previously, the cytoplasmic domain of the human proEGF transmembrane region was reported as a novel suppressor of human thyroid carcinoma cell motility and cathepsin L-mediated elastinolytic invasion [34]. We observed that urinary proEGF levels were decreased only when the tumor was located in transitional cells, including bladder cancer and TCC-type kidney cancer subgroups. Interestingly, urinary proEGF levels were not significantly altered in RCC-type kidney cancer patients with tumors located in renal cells. Therefore, proEGF might be a useful clinical indicator of tumor location in urological carcinomas. Verification of this will also require additional, larger-scale studies.

4.2. *Pathway analysis of the bladder cancer urine proteome*

Using MetaCore software to interpret global changes in the urine proteome caused by bladder cancer, we identified several pathways that were altered, including most notably

Table 5 – Summary of statistical analyses of the efficacy of SAA4 and ProEGF urine levels in detecting kidney cancers.

Protein name	Hernia vs. kidney cancer (TCC + RCC)		Hernia vs. kidney cancer (TCC)		Hernia vs. kidney cancer (RCC)		Kidney cancer (RCC vs. TCC)		Bladder cancer vs. kidney cancer (TCC)		Bladder cancer vs. kidney cancer (RCC)	
	p-value	AUC	*p*-value	AUC	*p*-value	AUC	*p*-value	AUC	*p*-value	AUC	*p*-value	AUC
SAA4	**<0.001** (n = 98)	**0.867**	**<0.001** (n = 80)	**0.934**	**<0.001** (n = 87)	**0.826**	**0.005** (n = 29)	**0.818**	**<0.001** (n = 116)	**0.831**	0.497 (n = 123)	0.550
ProEGF	0.978 (N = 87)[a]	0.502	0.342 (N = 69)[a]	0.591	0.502 (N = 76)[a]	0.447	0.088 (N = 29)	0.692	0.085 (N = 116)	0.658	**<0.001** (N = 123)	**0.824**
Panel (SAA4 + ProEGF)	**<0.001** (n = 87)	**0.878**	**<0.001** (n = 69)	**0.966**	**<0.001** (n = 76)	**0.831**	**<0.001** (n = 29)	**0.934**	**0.001** (n = 116)	**0.799**	**<0.001** (n = 123)	**0.832**

Statistically significant values (*p* value ≤0.050 or AUC values >0.7) are highlighted in bold.
Measured by Western blot analyses based on constant amount of total urine protein.

the immune-response/alternative complement and blood-coagulation pathways (Supplemental Fig. 5A–B). Of the 39 network objects (*i.e.*, the molecules that comprise the network) for the two pathways, 19 and 18 were quantified as differential proteins in immune response and blood-coagulation pathways, respectively. Studies have shown that the complement system is a major effector of the humoral branch of the immune system, acting to protect the host from microorganisms [35]. In the operation of this system, larger fragments resulting from cleavage of a component bind to targets near activation sites, whereas smaller fragments diffuse from the site and may initiate localized inflammatory responses by binding to specific receptors. Complement fragments interact with each another to form functional complexes. Multiple complement components interact sequentially to form a macromolecular structure called membrane attack complex. This complex creates pores in the cell membrane and induces cell lysis [36–38]. The hemostatic system maintains the fluidity of blood under normal conditions and responds to vessel injury by rapidly forming a clot consisting of platelets and fibrin. Thrombin generated during propagation of both cascades converts fibrinogen to a fibrin network. Thrombin also activates coagulation factor XIII, which is a transglutaminase that stabilizes the clot through covalent cross-linking of fibrin [39]. The fibrin clot is ultimately resorbed during fibrinolysis. Plasmin, generated from plasminogen, is the major fibrinolytic protease. Conversion of plasminogen to plasmin could be triggered by both tissue plasminogen activator and the plasminogen activator, urokinase (PLAU). Under certain conditions, plasma kallikrein is also capable of stimulating plasmin generation [40]. Both blood coagulation and fibrinolysis are under the fine control of various regulators. Our iTRAQ study indicates that blood-coagulation and immune-response pathways are likely simulated during bladder tumor development and invasion. The proteins involved in these pathways are probably secreted into urine, a proximal fluid for the bladder. Therefore, most of the identified proteins associated with the two pathways showed increased levels in the bladder cancer urine proteome.

Another interesting pathway that was significantly altered ($p = 2.49 \times 10^{-4}$) is the cell adhesion–PLAU signaling pathway (Supplemental Fig. 5C). The binding of PLAU to its glycosylphosphatidylinositol-anchored PLAU receptor (PLAUR) mediates a variety of functions, including inflammation, vascular homeostasis, and tissue repair [41]. PLAU plays an important role in the regulation of migration and cell adhesion during tissue remodeling, and activates intracellular signaling upon binding to certain receptors on the cell surface [42–44]. In our iTRAQ data, urinary levels of PLAU were decreased in all bladder cancer subgroups. Several previous investigations have reported an association between PLAU and bladder cancer [45–47]. These studies showed that PLAU levels in cancer tissues are significantly lower in low-grade and non-invasive cancers than in advanced cancers, suggesting PLAU as a useful prognostic marker of recurrence and progression of superficial bladder cancer [45,46]. Lin and co-workers identified PLAU protein in the culture media of bladder cancer cell lines and reported that pro-PLAU was present at low levels or was absent in the urine of patients with high-grade, advanced-stage bladder tumors [47]. They speculated that pro-PLAU may be more readily converted to PLAU in cancer cells, resulting in the subsequent loss of pro-PLAU in the urine of cancer patients [47]. This inference is consistent with the notion that tissue and body fluid proteomes may not always be congruent due to biological transformations. The clinical significance of PLAU in relation to bladder tumors warrants further study.

5. Conclusions

In this study, we evaluated the reproducibility and applicability of two different approaches for abundant-protein-depletion in human urine. An immuno-based MARS Hu-14 depletion column was selected for coupling with isobaric labeling and multiple-dimensional LC to discover bladder cancer biomarkers using a retrospective cohort of urine samples. Six apolipoproteins and four additional proteins (APOA4, TIM, SAA4, and ProEGF) were verified using Bio-plex assays or Western blot analyses. All six apolipoproteins were able to differentiate bladder cancer from hernia with statistical significance. Additionally, the combination of SAA4 and proEGF exhibited higher diagnostic capacity (AUC = 0.80 and $p < 0.001$) than either marker alone. A MetaCore analysis of biological networks revealed that immune-response/alternative complement and blood-coagulation pathways were most notably altered in the urine proteome of bladder cancer patients. This study confirmed the clinical significance of alterations in the urine proteome with respect to urological tumors and identified several urinary biomarkers for detecting bladder cancer. Further experiments will be required to validate clinical utility using independent sets of clinical samples and a prospective study design.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jprot.2013.04.024.

Acknowledgments

This work was supported by a grant to Chang Gung University from the Ministry of Education of Taiwan (EMRPD190041), Republic of China, as well as by grants from the Chang Gung Memorial Hospital (CMRPG371253, CMRPD190602, CLRPD190011), the National Health Research Institutes (NHRI-EX101-10015BI) and the National Science Council of Taiwan, Republic of China (NSC99-2320-B-182-017-MY3, NSC99-2923-B-182-002-MY2).

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