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Discovery of Apo-A1 as a potential bladder cancer biomarker by urine proteomics and analysis

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ABSTRACT

Bladder cancer is clinically characterized by high recurrent rate and poor prognosis and thereby patients need regular re-examinations which are invasive, unpleasant, and expensive. A noninvasive and less expensive method for detecting and monitoring bladder cancer would thus be advantageous. In this study, by using the two-dimensional electrophoresis (2-DE) approach with subsequent mass spectrometry (MS), we demonstrated the increased expression of apolipoprotein-A1 (Apo-A1) in individual urine from patients with bladder cancer, which was confirmed by Western blot results. A further analysis of the urinary Apo-A1 levels by an enzyme-linked immunosorbent assay yielded results that were consistent with the Western blot, and suggested Apo-A1 could provide diagnostic utility to distinguish patients with bladder cancer from healthy controls at 19.21 ng/ml. Further validation assay in a larger number of urine samples (n = 379) showed that Apo-A1 could be used as a biomarker to diagnosis bladder cancer with a sensitivity and specificity of 89.2% and 84.6% respectively. Moreover, the application of exfoliative urinary cytology in combination with the urine Apo-A1 detection could significantly increased the sensitivity in detecting bladder cancer. Our data showed a significant relationship of expressed Apo-A1 was established between bladder cancer and normal controls. Apo-A1 could be a potential biomarker for the diagnosis of bladder cancer.

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1. Introduction

Bladder cancer is the second most common urological cancer, clinically characterized by high recurrent rate and poor prognosis [1]. It is estimated that bladder cancer will account for 73,510 new cases of cancer and 14,880 cancer-related deaths in the United States during 2012 [2]. Therefore, bladder cancer remains a focus in cancer research. Generally, within 5–15 years after initial resection, the recurrence rate is as high as 60–85% [3]. Moreover, approximately 20% of the recurred bladder cancer cases can develop into muscle-invasive tumors [4].

At present, cystoscopy with cytology is the standard for diagnosing and monitoring bladder cancer. Cystoscopy is an invasive, relatively costly technique that may also be affected at times, particularly in cystitis cases [5,6]. Cytology is specific and noninvasive for diagnosing bladder carcinoma but less sensitive, particularly for detecting low-grade disease, which has limited the clinical relevance [7]. The invasiveness of cystoscopy and the limitations of cytology for detecting bladder cancer have generated interest in other simple, noninvasive diagnostic tools.

Proteomic patterns in body fluids gain increasing importance as a new tool for the identification of novel, highly sensitive diagnostic markers for the detection of cancer [8–10]. Proteomics determines disease-associated biomarkers by observing the expression patterns of proteins thereby distinguishing between normal and dysregulated processes. Two-dimensional electrophoresis (2-DE) followed by mass spectrometry (MS) has been wildly applied in differential proteomic studies of urine specimens for biomarker discovery [11,12]. One promising approach in the search for useful bladder cancer biomarkers is to study the urine proteome during the occurrence of the disease [13,14]. Urine is in direct contact with bladder epithelia cells and the changes of the composition, quantity, and quality may reflect the information that could represent the generation, development, and prognosis of the urinary

Abbreviations: 2-DE, two-dimensional electrophoresis; Apo-A1, apolipoprotein-A1; PMH, post medical history; IPG, immobilized pH gradient; ROC, receiving operating curve; AUC, area under the curve.

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tumor. Compared with the collection of plasma and other body fluid samples, the collection of urine is convenient and noninvasive. In the past years, several proteins in urine were researched as biomarkers for bladder cancer, such as orosomucoid, zinc-alpha$_2$-glycoprotein, fibrinogen β chain and α-1-antitrypsin [12,15].

The development of proteomics has provided us new tools in determining biomarkers for the detection and follow-up of bladder cancer. In this study, by using the 2-DE approach with subsequent MS, we demonstrated the increased expression and secretion of apolipoprotein-A1 (Apo-A1) protein in individual urine from patients with bladder cancer, which was further confirmed by Western blot analyses and enzyme-linked immunosorbent assay (ELISA). Moreover, the application of exfoliative urinary cytology in combination with the urine Apo-A1 detection could significantly increased the sensitivity and accuracy in bladder cancer detecting. These findings strongly suggest Apo-A1 could be a potential biomarker for bladder cancer detection and diagnosis.

2. Materials and methods

2.1. Study population

Patients with bladder cancer were enrolled at Second Hospital of Tianjin Medical University between 2011 and 2013. All the patients had histopathologically confirmed bladder cancer, and none had received chemotherapy or radiation before enrollment. Patients were confirmed without resent post medical history (PMH) and any symptom of kidney failure. Age-, sex-, and ethnicity-matched control subjects were healthy volunteers with no history or evidence of urological cancer who were recruited from the Medical Examination Center. Chronic urinary tract diseases were also excluded. This study was approved by the Ethics Committee of Second Hospital of Tianjin Medical University. The human samples were collected according to the Ethics Committee guidelines. The written informed consent was obtained from all subjects.

2.2. Urinary sample preparation

Approximately 50 ml of clean-catch, the first morning mid-stream voided urine from 10 healthy donors or 10 patients with bladder cancer was collected and immediately centrifuged at 1500*g*, at 4 °C for 10 min. The sample for Western blot and ELISA analysis was collected from the supernatant and stored at −80 °C. The left supernatant was further centrifuged at 12,000*g*, 4 °C for 10 min and the pellet was washed twice using 25% ethanol and then dissolved in solubilizing buffer which contained 7 M urea, 2 M thiourea, 4% CHAPS, 0.2% ampholytes, 65 mM DTT inorganic salts and any other interfering components were removed by using 2D Clean-Up Kit (Bio-Rad, Hercules, CA, USA). The protein concentration of urinary proteins was assessed with Bradford assay kits.

2.3. 2-DE analysis

Urinary protein samples (100 μg) were solubilized in 350 μl rehydration buffer containing 7 M urea, 2 M thiourea, and 4% CHAPS, 0.2% ampholytes (pH 3–10), 65 mM DTT, 2 mM TBP and 0.001% bromophenol blue. Solubilized samples were loaded onto the immobilized pH gradient (IPG) strips, 17 cm pH 4–7, for the first dimension. After equilibration, IPG gel strips were transferred onto 12% polyacrylamide gel and covered with 0.5% agarose gel for the second dimension. Polyacrylamide gels were stained with silver according to the procedures described previously [16]. Protein spots were quantified using PDQuest analysis software (Bio-Rad, Hercules, CA, USA). All experiments were repeated at least 3 times.

2.4. MS analysis

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) analysis was performed according to procedures described previously [11]. Briefly, protein spots were excised and destained by 25 mM ammonium bicarbonate and 50% CAN. An in-gel digestion was performed with 0.01 μg/μl trypsin in 25 mM ammonium bicarbonate for 15 h at 37 °C. The supernatants were collected, and tryptic peptides were extracted from the gel sequentially with 5% TFA at 40 °C for 1 h and with a solution of 2.5% TFA and 50% CAN at 30 °C for 1 h. The extracts were dried completely and then dissolved in 0.5% TFA. 1 μl of the peptide solution mixed with 1 μl of matrix (4-hydroxy-a-cyanocinnamic acid in 30% CAN and 0.1% TFA) were loaded on the target plate for MS analysis using a mass spectrometry 4700 (Applied Biosystems, Framingham, MA). The Homo sapiens subsets of the sequences in the Swiss-Prot and NCBI nonredundant protein sequence databases were utilized for MASCOT searches.

2.5. Western blot

50 μg urinary proteins were loaded on 10% SDS–PAGE gels and subsequently blotted onto PVDF membrane. After blocking, the membrane was incubated with primary antibodies against Apo-A1 (AAH05380) for 2 h. Following washing, the membrane was exposed to secondary antibodies conjugated with HRP for 30 min. The antigen–antibody complexes were detected using a chemiluminescence detection kit (ECL; Pierce, Thermo Fisher Scientific, Courtaboeuf, France) and visualized by autoradiography. The X-ray films were digitalized for subsequent densitometric quantification of the bands using the morphometric program Optimas (Optimas Corporation, Seattle, USA).

2.6. Enzyme linked immunosorbent assay (ELISA)

Soluble urinary Apo-A1 concentrations were measured by ELISA, using Quantikine Immunoassay Kits (CUSABIO, Wuhan, CHN) according to the manufacturer's protocol. After the development of the colorimetric reaction, the OD at 450 nm was quantified by an eight-channel spectrophotometer, and the OD readings were converted to nanograms per milliliter (ng/ml) on the basis of the standard curves obtained with Apo-A1 standard preparation in assay. Each sample was tested in duplicate. Apo-A1 concentrations were represented as mean ± SD.

2.7. Exfoliative urinary cytology

Acridine orange staining was used in this study. The first time fresh, mid-stream morning urine about 30–50 ml were collected and centrifuged at 2000 rpm for 10 min. Urine sediments were suspended and fixed with fixation fluid (prepared with glacial acetic acid, chloroform and dehydrated alcohol at a 1:3:6 ratio). After recentrifuging, urine sediments were dropped onto slides and soaked in acridine orange solution for 3 min and washed with PBS, then soaked in 0.1% calcium chloride solution for 3 min, and rewashed with PBS. Eventually, the slides were observed via fluorescence microscopy.

2.8. Immunohistochemical staining

Formalin-fixed, paraffin-embedded tissue sections obtained from patients who had bladder cancer as well as normal bladder tissue sections were used for immunohistochemical analyses. Briefly, each section was deparaffinized in descending alcohol, then treated with normal rabbit serum to block the unspecific background for 30 min. The sections were incubated with primary

antibodies against Apo-A1 (AAH05380, 1:200 dilution) at 4 °C overnight, washed 3 times with PBS, and exposed to HRP-conjugated secondary antibodies for 30 min. The binding was detected using DAB as the substrate. Sections were counterstained with hematoxylin. The intensity of Apo-A1 staining was assessed by light microscopy.

2.9. Statistical analysis

The data expressed as mean value ± SD. The two-tailed distribution *t* test was used. Receiving operating curve (ROC) analysis was used to define the most optimal diagnostic cutoff as well as the diagnostic performance given by the area under the curve (AUC), estimating its 95% confidence interval at an optimal cutoff. Statistical analyses were performed using the SPSS statistical package (version 13.0).

3. Results

3.1. Apo-A1 express is increased in urine of patients with bladder cancer

Urine proteins from patients with bladder cancer and healthy controls were resolved by 2-DE individually. 2-DE images stained by silver revealed expression profiles of urinary proteins in the bladder cancer and normal control groups were similar but not identified (data not shown). Of those differentially expressed protein dots, two spots about 28 kDa in pH range 5–6 attracted our attention, which obviously expressed at an increased level in bladder cancer than in healthy control (Fig. 1) and were later identified both as Apo-A1 by MS. Information of Apo-A1 are presented in Table 1. To analyze further knowledge about Apo-A1, we used the gene ontology (GO) platform to obtain much more information with aspects of molecular function and biologic process (Table 1).

3.2. Apo-A1 expression is verified in individual urine sample by Western blot analysis

Verification of Apo-A1 to have higher abundance in urine from bladder cancer patients, and to prove the MS results, involved Western blot using antibodies against Apo-A1. Urine samples from 6 patients and 6 healthy controls were randomly selected for WB and the results were shown in Fig. 2. Significantly higher protein abundance of Apo-A1 in bladder cancer patients compared with controls was confirmed.

3.3. Assessment of the diagnostic efficacy of urine Apo-A1 in detecting bladder cancer

The potential clinical utility of Apo-A1 at discriminating patients with bladder cancer from healthy individuals was analyzed. Urine samples categorization was based on histopathological confirmation. Apo-A1 concentration were measured in individual urine samples from controls ($n = 40$) and bladder cancer patients ($n = 40$) using a commercial ELISA. ELISA results as demonstrated in Fig. 3 revealed that urine Apo-A1 concentrations are extremely high in bladder cancer group compared with healthy controls ($P = 0.000$). At a cutoff value of 19.21 ng/ml, Apo-A1 was able to

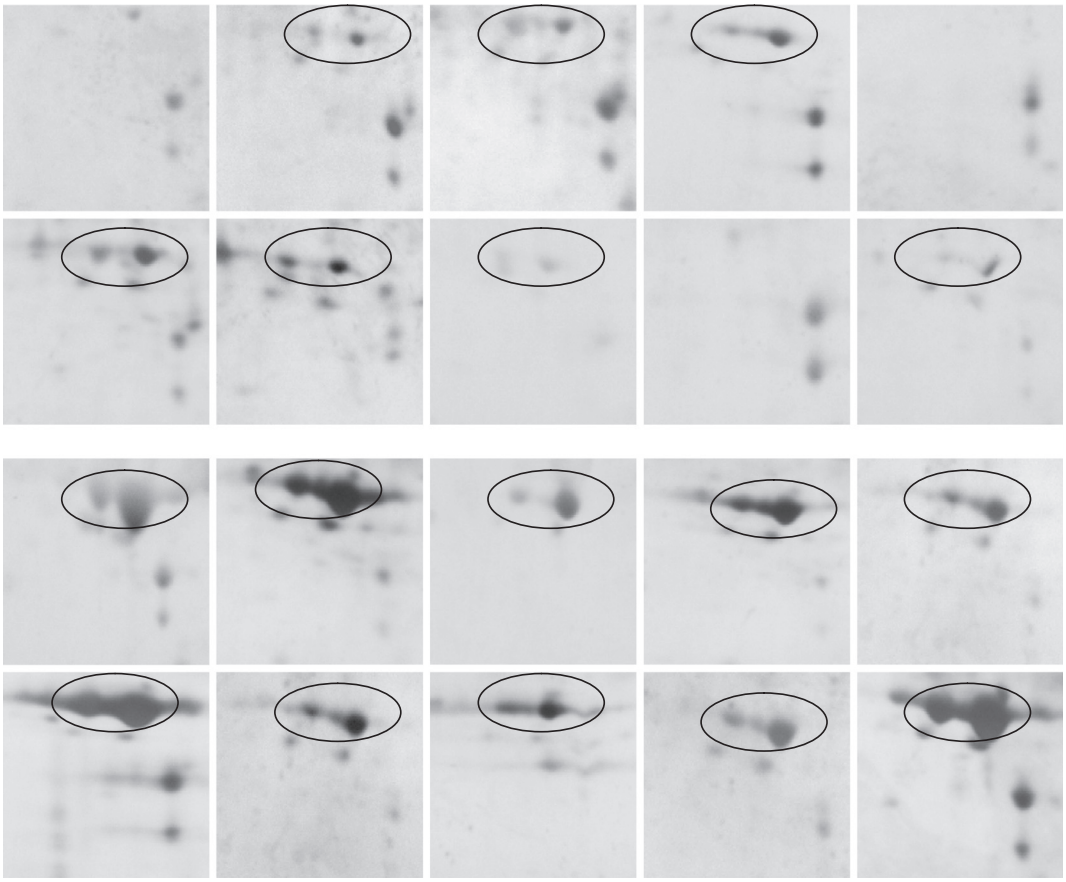

Fig. 1. 2-DE protein patterns of Apo-A1 in urine samples from patients with bladder cancer and healthy individuals. Urine proteins from healthy controls (upper) or patients with bladder cancer (lower) was resolved by 2-DE. The gels were stained by sliver. Two spots that overexpressed in bladder cancer compared with healthy controls were excised individually and digested in gel with trypsin, which were identified as Apo-A1 by MS.

Table 1
Informations of differentially expressed urinary protein Apo-A1 in bladder cancer identified by MS.

Accession No.	gi|490098
Protein Name	Apo-A1
Mr (kDa)	28.1
PI	5.27
Total ion score C.I.%	100
Expression	Up
Molecular function	Apo-A1 receptor binding; beta-amyloid binding; cholesterol binding; cholesterol transporter activity; enzyme binding; high-density lipoprotein receptor binding; identical protein binding; phosphatidylcholine sterol Oacyltransferase activator activity; phospholipid binding
Biologic process	Cdc42 protein signal transduction; G protein coupled receptor protein signaling pathway; cholesterol efflux; cholesterol homeostasis; cholesterol import; high-density lipoprotein particle assembly; high-density lipoprotein particle clearance; high-density lipoprotein particle remodeling; negative regulation of cytokine; secretion during immune response; negative regulation of interleukin-1β secretion; negative regulation of very-low-density lipoprotein particle remodeling; phosphatidylcholine biosynthetic process; phospholipids efflux; positive regulation of cholesterol esterification; positive regulation of hydrolase activity; protein stabilization; reverse cholesterol transport

discriminate bladder cancer from controls with a sensitivity and specificity of 92.5% and 80.0%, respectively.

3.4. Validation of the diagnostic efficacy of urine Apo-A1 in detecting bladder cancer

An additional study using a larger set of urine specimens was performed to confirm the utility of Apo-A1 in urine as a bladder cancer biomarker. Urine samples ($n = 379$) were collected randomly and numbered orderly. Apo-A1 level were measured using ELISA and the diagnosis was made according to the optimal cutoff value suggested by ROC curve in Fig. 3. Final clinical diagnosis was made based on histopathological results. Our data showed Apo-A1 can diagnose bladder cancer with a sensitivity and specificity of 89.2% and 84.6%, respectively (Table 2A).

3.5. Assessment of the assistant diagnostic value of urine Apo-A1 to urinary cytology

Acridine orange staining (Supplemental Fig. 1) was used to value the sensitivity and specificity of exfoliative urinary cytology examination. Single positive of exfoliative urinary cytology or ApoA1 detection and double positive of these two indexes were all considered as having bladder cancer. Single exfoliative urinary cytology results presented the sensitivity and specificity as 72.2% and 90.4%, respectively (Table 2B). Surprisingly, when urinary cytology was used in combination with Apo-A1 examination, the sensitivity was increased to 93.7% (Table 2C).

3.6. Apo-A1 expression is not detectable neither in morphologically normal urotheila nor in bladder cancer tissue by immunostaining

Urine is in direct contact with bladder epithelia cells and proteins released from bladder tumor cells may be enriched in urine samples, we further investigated if Apo-A1 is expressed in bladder tissue. To our surprise, Apo-A1 detected in urine from bladder cancer patients was expressed neither in randomly selected bladder cancer tissue nor in morphologically normal bladder tissue (Fig. 4).

4. Discussion

Several biomarkers indicated changes in the expression of urinary proteins associated with increased risk have been identified for the detection of bladder cancer by using proteome analysis [9–12,17,18]. In our previous study, we identified several urine proteins with increased or decreased levels between pooled urine samples from healthy controls and patients with bladder cancer

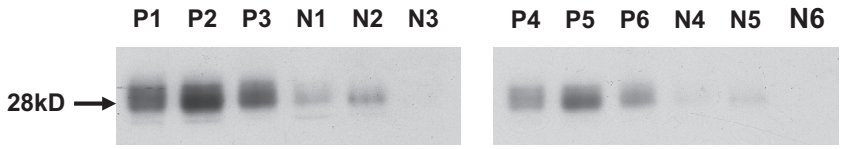

Fig. 2. Verification of Apo-A1 expression in individual urine sample by Western blot. Apo-A1 expression in individual urine samples from healthy controls, or patients with bladder cancer were determined by Western blot. 50 μg urinary protein samples from 6 patients with bladder cancer (P1–6) and 6 healthy controls (N1–6) was loaded on 10% SDS–PAGE gels, blotted onto PVDF membrane and probed with monoclonal antibodies against Apo-A1.

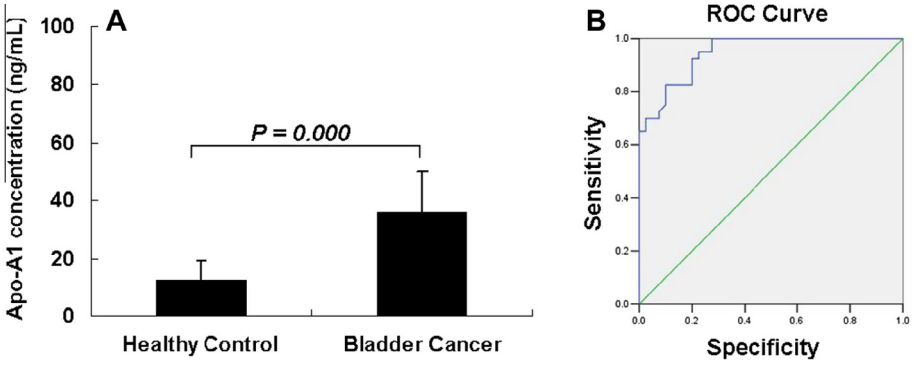

Fig. 3. Apo-A1 as a biomarker for predicting bladder cancer. (A) Quantitative proteomic analysis of Apo-A1 in urine samples by ELISA. (B) Urinary Apo-A1 distinguishes bladder cancer on independent series of urine samples of patients with bladder cancer and controls. ROC curve of urinary Apo-A1 as a detection marker for bladder cancer was based on a series of 80 urine samples. Among these, 40 had cancer-positive. The optimal cutoff was 19.21 ng/ml, and the AUC obtained was 0.948 (95% CI 0.906–0.990).

Table 2
Value of urinary cytology and Apo-A1 in the diagnosis of bladder cancer.

Group	Pathologic diagnosis	Apo-A1 level	
		>19.21 ng/mL	<19.21 ng/mL
A			
Bladder cancer	223	199	24
Non-bladder cancer	156	24	132

Group	Pathologic diagnosis	Urinary cytology	
		Positive	Negative
B			
Bladder cancer	223	161	62
Non-bladder cancer	156	13	141

Group	Pathologic diagnosis	Apo-A1 level & urinary cytology	
		Positive	Negative
C			
Bladder cancer	223	209	14
Non-bladder cancer	156	25	131

by using the proteomics technology [11]. Among those identified proteins, a protein about 28 kDa was shown significantly increased expression in bladder cancer group. Considering the expression levels in pooled samples might not be able to reflect the level in individual sample, we analyzed this increased protein expression using a series of independent urine samples in present study and it was confirmed with differentially increased expression in urine samples of bladder cancer than healthy controls. Followed MS analysis confirmed the protein is Apo-A1, which was consistent with our previous study. These results were further confirmed by Western blot, suggesting that a high level of urine Apo-A1 could be a potential biomarker for bladder cancer.

Apo-A1 is a major high-density lipoprotein (HDL) component in plasma, which constitutes approximately 70% of the apolipoprotein content of HDL particles [19]. Several previous studies showed changes in serum lipids and lipoprotein in cancer patients, some of which are associated with cancer progression. Apo-A1 was reported decreased expression in the serum of patients with pancreatic cancer, colorectal cancer and ovarian cancer [20,21]. Controversial observations were also reported in a variety of malignant tumors. Serum Apo-A1, which may indirectly promote tumor survival through kinase activation, were found to be overexpressed in patients with recurrent head and neck squamous cell carcinoma [22]. A significant association between up-regulated lipoprotein and the presence and stage of lung cancer was observed and the possible role of lipoproteins in the development of tumor angiogenesis was discussed [23].

Recent attention has focused on the level of lipoproteins in urine of patients with bladder cancer since proteomics analysis was used for urinary biomarker discovery. Increased level of Apo-A1 was reported in urine samples from patients with bladder cancer [24,25]. However, these studies were mostly stopped at identification step without further clinical utility analysis. In present study, after identification using 2-DE followed by MS in a series of individual urine samples, quantitative measurement ELISA was performed to evaluate whether Apo-A1 could be utilized as a diagnostic tool for bladder cancer. The data revealed that urine Apo-A1 are extremely high in cancer groups compared with normal controls. ROC analyses rendered a diagnostic accuracy of 0.948 at a cutoff value of 19.21 ng/ml. Our findings indicate that Apo-A1 might represent a novel urine marker for detection of bladder cancer. Although these results are promising, a further validation analysis using a larger set of urine samples will be required to confirm the utility of Apo-A1 in urine as a bladder cancer biomarker. Our results from 379 urine samples further confirmed above findings that Apo-A1 can distinguish between bladder cancer patients and normal controls with high sensitivity and specificity, which suggests the potential diagnostic application of urinary Apo-A1 for bladder cancer.

Currently, many bladder cancer biomarkers are being researched, but only a few are commercially available approved products. Until now, none of the currently available urinary markers have met the standards in terms of sensitivity and specificity for replacing the combination of cystoscopy and cytology. Nevertheless, the clinical value of these urinary biomarkers should not be ignored completely. Proper applications like as an assisting technique might be helpful for diseases monitoring. Exfoliative urinary cytology, as the second criterion standard for diagnosis of bladder cancer, is still wildly used clinically, though having a median sensitivity which limited the clinical relevance [26]. We would like to investigate the combined diagnostic value of urine Apo-A1 and cytology in this study. Exfoliative urinary cytology results from 379 cases presented the sensitivity and specificity of bladder cancer detection as 72.2% and 90.4%, respectively. When we combined exfoliative urinary cytology with Apo-A1 examination, the sensitivity was increased to 93.7% according to the combinate diagnostic standards we defined in methods. Though the specificity was slightly lower compared with single cytology application, the combination could help avoid missed diagnosis, which apparently will benefit the patients. These findings strongly suggest that urine Apo-A1 could be used as an auxiliary index for exfoliative urinary cytology in detecting bladder cancer.

Though lipoproteins was considered to play possible roles in indirectly promoting tumor survival through kinase activation, or in the development of tumor angiogenesis [22,23], the association of changes in lipoprotein levels and lipoprotein metabolic pathways

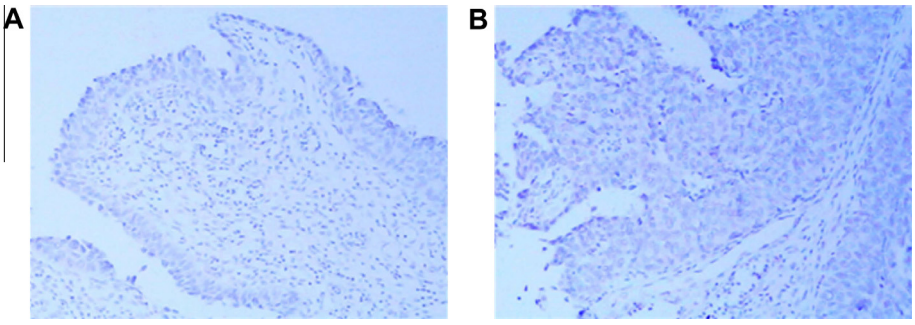

Fig. 4. Immunostainings of Apo-A1 protein in morphologically normal urotheila and bladder cancer tissue. Formalin-fixed, paraffin-embedded tissue sections of morphologically normal urotheila (A) and bladder cancer tissue (B) were incubated with primary antibodies against Apo-A1 at 4 °C overnight, following with HRP-conjugated secondary antibodies incubation. The binding was detected using DAB as the substrate.

with cancer progression, and differences in the lipoprotein-related proteome between bladder tissue and urine remain unclear and warrant in-depth investigation. Exploring the likely source of Apo-A1 which may help to understand this potential biomarker was of interest. Urine is in direct contact with bladder epithelia cells, which may give rise to bladder cancer, proteins released from bladder tumor cells may be enriched in urine samples. Therefore, tumor cells were considered the most likely source of Apo-A1. Surprisingly, Apo-A1 detected in urine from bladder cancer patients were expressed neither in bladder cancer tissue nor in morphologically normal bladder tissue, our results were supported by the information provided by the HPA (http://www.proteinatlas.org). This finding is of great biological interest since it implies that the detection of Apo-A1 in urine from patients with bladder cancer cannot be explained by their expression in tumor cells. So, where are urine APO-A1 proteins from? To answer this question, further researches will be performed by our group.

Noninvasive detection of proteins secreted from bladder tumor or normal cells in urine as biomarkers may be useful for clinical application in bladder cancer diagnosis. In this study, we identified a high level of Apo-A1 as a novel candidate urine biomarker for bladder cancer diagnosis. Further studies investigating the source of urine Apo-A1 and verifying the correlation between Apo-A1 and the clinical outcome of bladder tumors are needed to assess whether their detection in urine can be used as biomarkers for urinary bladder cancer.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.03.053.

References

[1] B. Jacobs, C.T. Lee, J.E. Montie, Bladder cancer in 2010: how far have we come?, CA Cancer J Clin. 60 (2010) 244–272.
[2] R. Siegel, D. Naishadham, A. Jemal, Cancer statistics 2012, CA Cancer J. Clin. 62 (2012) 10–29.
[3] B.W. van Rhijn, H.G. van der Poel, T.H. van der Kwast, Urine markers for bladder cancer surveillance: a systematic review, Eur. Urol. 47 (2005) 736–748.
[4] A. Karl, S. Tritschler, D. Zaak, et al., Diagnostic procedure for bladder cancer. Standards and current developments, Urologe A 49 (2010) 1303–1311.
[5] D. Mitropoulos, A. Kiroudi-Voulgari, P. Nikolopoulos, et al., Accuracy of cystoscopy in predicting histologic features of bladder lesions, J. Endourol. 19 (2005) 861–864.
[6] M.N. van der Aa, E.W. Steyerberg, C. Bangma, et al., Cystoscopy revisited as the gold standard for detecting bladder cancer recurrence. Diagnostic review bias in the randomized, prospective CEFUB trial, J. Urol. 183 (2010) 76–80.
[7] E.B. Avritscher, C.D. Cooksley, H.B. Grossman, et al., Clinical model of lifetime cost of treating bladder cancer and associated complications, Urology 68 (2006) 549–553.
[8] T. Krüger, J. Lautenschläger, J. Grosskreutz, et al., Proteome analysis of body fluids for amyotrophic lateral sclerosis biomarker discovery, Proteomics Clin. Appl. 7 (2013) 123–135.
[9] S.L. Wood, M.A. Knowles, D. Thompson, et al., Proteomic studies of urinary biomarkers for prostate, bladder and kidney cancers, Nat. Rev. Urol. 10 (2013) 206–218.
[10] S.I. Mohammed, M. Rahman, Proteomics and genomics of urinary bladder cancer, Proteomics Clin. Appl. 2 (2008) 1194–1207.
[11] H. Li, C. Li, H. Wu, et al., Identification of Apo-A1 as a biomarker for early diagnosis of bladder transitional cell carcinoma, Proteome Sci. 9 (2011) 21.
[12] S. Irmak, D. Tilki, J. Heukeshoven, et al., Stage-dependent increase of orosomucoid and zinc-alpha$_2$-glycoprotein in urinary bladder cancer, Proteomics 5 (2005) 4296–4304.
[13] M. Pejcic, S. Stojnev, V. Stefanovic, Urinary proteomics—a tool for biomarker discovery, Ren. Fail. 32 (2010) 259–268.
[14] W. Mullen, The human urinary proteome: combinational approaches to comprehensive mapping, Expert Rev. Proteomics 9 (2012) 375–377.
[15] M. Lindén, S.B. Lind, C. Mayrhofer, et al., Proteomic analysis of urinary biomarker candidates for nonmuscle invasive bladder cancer, Proteomics 12 (2012) 135–144.
[16] C.R. Merril, D. Goldman, S.A. Sedman, et al., Ultrasensitive stain for proteins in polyacrylamide gels shows regional variation in cerebrospinal fluid proteins, Science 211 (1981) 1437–1438.
[17] C.L. Chen, T.S. Lin, C.H. Tsai, et al., Identification of potential bladder cancer markers in urine by abundant-protein depletion coupled with quantitative proteomics, J. Proteomics 85 (2013) 28–43.
[18] L. Grau, J.L. Luque-Garcia, P. González-Peramato, et al., A quantitative proteomic analysis uncovers the relevance of CUL3 in bladder cancer aggressiveness, PLoS One 8 (2013) e53328.
[19] P.S. Chetty, L. Mayne, S. Lund-Katz, et al., Helical structure and stability in human apolipoprotein A-I by hydrogen exchange and mass spectrometry, Proc. Natl. Acad. Sci. USA 106 (2009) 19005–19010.
[20] M. Ehmann, K. Felix, D. Hartmann, et al., Identification of potential markers for the detection of pancreatic cancer through comparative serum protein expression profiling, Pancreas 34 (2009) 205–214.
[21] H. Dieplinger, D.P. Ankerst, A. Burges, et al., Afamin and apolipoprotein A-IV: novel protein markers for ovarian cancer, Cancer Epidemiol. Biomarkers Prev. 18 (2009) 1127–1133.
[22] C.G. Gourin, W. Zhi, B.L. Adam, Proteomic identification of serum biomarkers for head and neck cancer surveillance, Laryngoscope 119 (2009) 1291–1302.
[23] H.H. Yang, X.F. Chen, W. Hu, et al., Lipoprotein(a) level and its association with tumor stage in male patients with primary lung cancer, Clin. Chem. Lab Med. 47 (2009) 452–457.
[24] T. Lei, X. Zhao, S. Jin, et al., Discovery of potential bladder cancer biomarkers by comparative urine proteomics and analysis, Clin. Genitourin. Cancer 11 (2013) 56–62.
[25] Y.T. Chen, C.L. Chen, H.W. Chen, et al., Discovery of novel bladder cancer biomarkers by comparative urine proteomics using iTRAQ technology, J. Proteome Res. 9 (2010) 5803–5815.
[26] R. Liu, Z. Tian, J. Wang, et al., Evaluation of acridine orange fluorescence in exfoliative urinary cytology for diagnosing bladder carcinoma, Int. Urol. Nephrol. 44 (2012) 1375–1382.