Urological Science 27 (2016) 250–257

Contents lists available at ScienceDirect

Urological Science

journal homepage: www.urol-sci.com

Original article

Searching urinary tumor-associated proteins for bladder transitional cell carcinoma in southwestern Taiwan using gel-based proteomics

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ARTICLE INFO

Article history:
Received 21 March 2015
Received in revised form
18 August 2015
Accepted 24 August 2015
Available online 10 December 2015

Keywords:
two-dimensional gel electrophoresis
arsenic
bikunin
bladder transitional cell carcinoma
LC/MS/MS
perlecan

ABSTRACT

Background and purpose: We try to search for specific serum or urinary biomarkers for the early detection, follow-up, and prediction of tumor recurrence, progression, and clinical outcome is a difficult task in individuals with bladder cancer.
Materials and methods: In this study, urinary samples were dialyzed to remove any interfering molecules and concentration by lyophilization. The urinary proteome maps of 10 healthy volunteers and 10 bladder transitional cell carcinoma (BTTC) patients were explored through two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) coupled with mass spectrometry. With no fractionation, the proteome maps acquired in this study likely represented the total urinary proteins.
Results: Comparative proteomics indicated that six proteins were down-regulated and five proteins were up- regulated in BTCC patients as compared with normal. The down-regulated spots were identified as human haptoglobin precursor, human heparan sulfate proteoglycan perlecan, inter-alpha-trypsin inhibitor heavy chain H4 precursor, and AMBP protein precursor. The up-regulated spots were identified as peroxiredoxin 2, heparan sulfate proteoglycan perlecan, protease serine 1 fragment and AMBP protein precursor. Most of these de-regulated proteins were extracellular matrix–associated proteins, which may play roles in regulating the immune response, signal transduction and tumor invasions.
Conclusion: In this paper, 11 de-regulated proteins were observed in the urinary specimens of BTTC patients from the southwestern coast of Taiwan where Blackfoot disease is endemic and the unusually high incidence of BTTC in this area might attribute to high arsenic content in the drinking water. It is possible that long-term arsenic-induced alteration of these de-regulated proteins, most of which were extracellularmatrix – (ECM) related proteins which may play roles in regulating the immune response, signal transduction and tumor invasions, might be involved in BTTC development in southwestern Taiwan.

1. Introduction

The most prevalent cancer of the urinary tract is tumor of the urinary bladder. It includes a broad range of histological heterogeneous tumor types arising mostly from the urothelium lining of the urinary bladder and ureters, including bladder transitional cell carcinoma (BTCC), squamous cell carcinoma, adenocarcinoma, and other less frequent lesions.[1] More than 90% of the bladder tumors are diagnosed as BTCC and the majority of BTCC (70%) are recognized as superficial papillary lesions (stage pTa, T1).[1] Recurrences

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http://dx.doi.org/10.1016/j.urols.2015.08.009

are commonly observed in superficial tumors but few cases will progress to higher grade and/or stage or muscle invasion.[2]

Currently, cytoscopy combined with urine cytology is routinely used for the diagnosis of BTCCs. Urine cytology is mainly used for the diagnosis and follow-up of patients with malignancy. It is noninvasive and offers high specificity as well as satisfactory sensitivity for the detection of high-grade BTCCs but lacks sensitivity for low-grade lesions. However, cytoscopy has high specificity but is invasive, expensive, and occasionally inconclusive in cases of high-grade flat tumors and cystitis.[3] Because of the aforementioned blemish, more noninvasive and accurate biomarkers are required for the diagnosis and prognosis of bladder cancer.

To date, many urinary BTCC biomarkers have been reported. Among these, bladder tumor antigen (BTA Stat and BTA TRAK), nuclear matrix protein 22 (NMP-22 enzyme-linked immunosorbent assay detection kit), and tumor-associated antigens M344, 19A211, and LDQ19 (ImmunoCyt fluorescence test), fibrinogen-fibrin degradation products (FDP test), and UroVysion fluorescent *in situ* hybridization assay have been approved by the United States Food and Drug Administration for diagnostic purposes.[4] Other urinary tests such as survivin test, hyaluronidase (HA-Hase test), BCLA-4, and cytokeratin (Ck)-20 are still being investigated.[4,5] Some of these assays have demonstrated higher sensitivity than the conventional urine cytology, but most display lower specificity. In general, these assays are more expensive and may only be performed by experienced personnel. Thus, searching for specific serum or urinary biomarkers for the early detection, follow-up, and prediction of tumor recurrence, progression, and clinical outcome is a difficult task in individuals with bladder cancer.

In this study, two-dimensional gel electrophoresis (2-DE) was used to establish the urinary proteome maps of the patients with BTCC from the southwestern coast where blackfoot disease, which is caused by the consumption of water contaminated by arsenic, is endemic. Coupled with tandem mass spectrometry (MS/MS), two-dimensional gel comparisons between the urinary protein profiles of healthy persons and patients with BTCC were implemented to find the differentially expressed protein present in the patient urine and these dysregulated proteins might be the potential biomarkers for diagnosis or prognosis in the future. The results of proteomic comparisons revealed that several differentially expressed proteins existed in the urine of patients with BTCC from the southwestern coast of Taiwan and most of these proteins belonged to the extracellular matrix proteins.

Table 1
Clinical information on patients with bladder transitional cell carcinoma and normal donors.

Case No.	Sex/Age (y)	Stage	Grade
Patients			
CM001	F/83	T1	II
CM002	F/77	T2	II
CM003	F/65	T1	II
CM101	M/84	T1	II
CM102	M/72	T1	II
CM103	M/73	T2	III
CM105	M/50	T1	III
CM106	M/77	T2	III
CM107	M/72	T2	III
CM004	F/65	T2	III
Healthy donors			
NM001	M/25	—	—
NM002	M/25	—	—
NM003	M/25	—	—
NM004	M/25	—	—
NM005	M/25	—	—
NM006	M/25	—	—
NF001	F/23	—	—
NF002	F/23	—	—
NF003	F/23	—	—
NF004	F/23	—	—

F = female; M = male.

2. Materials and methods

2.1. Urine from patients with BTCC

200-mL urine samples collected from each of 10 healthy individuals or 10 patients with BTCC at Chi-Mei Medical Center (Tainan, Taiwan), a southwestern medical center proximal to the blackfoot disease-endemic region in Taiwan, were immediately transferred to the laboratory, centrifuged at 9300*g* for 30 minutes at 15°C using Micromax RF (Thermo IEC) and the supernatant was frozen at −80°C for future proteomic analyses. An informed consent was obtained from each patient and healthy donors. The clinical information of the patients and control donors are listed in Table 1. The current study was approved by the Medical Research Committee of Chi-Mei Medical Center and complies with the guidelines for human study published by Administration of Health in Taiwan.

2.2. Preparation of human urinary protein samples

The frozen urine sample was lyophilized within 12 hours of arrival at the laboratory. Then the powder was dissolved in 30 mL sterile phosphate buffered saline and the resulting solution was dialyzed three times in the dialysis membrane (MWCO: 3.5 kDa; Pierce, Rockford, IL, USA) against 2 L of double distilled water. After dialysis, the solution was lyophilized again and the protein pellet was dissolved in 500 μL lysis buffer [7M urea, 2M thiourea, 100mM dithiothreitol (DTT), 4% (v/v) CHPAS, 40mM Tri-Base (pH 10), 1mM PMSF, and 1 Complete Mini protease inhibitor cocktail tablet (Roche, Diagnostics, Indianapolis, IN, USA) per liter] with shaking at room temperature for 1 hour. Then the lysate was centrifuged at 349,000*g* (Beckman Coulter, Fullerton, CA, USA) for 2 hours at 15°C in Type 90 Ti rotor and the supernatant was precipitated with a 2-D clean-up kit (Amersham Bioscience Corp. Piscataway, NJ, USA) according to the manufacturer's suggestion. The resulting protein lysate was measured by Bio-Rad D_C protein assay.

2.3. Isoelectric focusing

The pH 4-7, 18-cm immobibline dry strips (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) were rehydrated for 16 hours at 20°C with 300 μL rehydration buffer [7M urea, 2M thiourea, 4% (v/v) CHAPS, 2% (w/v) DTT, 0.5% (v/v) Immobilized pH gradient (IPG) buffer and trace of bromophenol blue]. After rehydration, 100 μg of urinary protein lysates prepared from each of healthy individuals or bladder cancer patients were cup-loaded onto the rehydrated gel strips with Ettan IPGphor Cup Loading Manifold (Amersham-Pharmacia Biotech Inc., Piscataway, NJ, USA). The proteins were then focused at 20°C at 50 V, 100 V, 200 V, 500 V, 1000 V, 5000 V, and 8000 V, respectively, with a total of 81,434 voltage-hours.

2.4. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

After isoelectric focusing, the gel strips were equilibrated in equilibration buffer [6M urea, 30% (v/v) glycerol, 2% (w/v) sodium dodecyl sulfate] containing 2% (w/v) DTT for 15 minutes and then in equilibration buffer containing 5% (w/v) iodoacetamide for an additional 15 minutes. The equilibrated gel was loaded onto the top of a 12.5% (w/v) polyacrylamide gel and sealed with 0.5% (w/v) agarose. The proteins were separated at 420 V using *BioRad Protean*