Comparative Tissue Proteomics of Microdissected Specimens Reveals Novel Candidate Biomarkers of Bladder Cancer*

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More than 380,000 new cases of bladder cancer are diagnosed worldwide, accounting for ~150,200 deaths each year. To discover potential biomarkers of bladder cancer, we employed a strategy combining laser microdissection, isobaric tags for relative and absolute quantitation labeling, and liquid chromatography-tandem MS (LC-MS/MS) analysis to profile proteomic changes in fresh-frozen bladder tumor specimens. Cellular proteins from four pairs of surgically resected primary bladder cancer tumor and adjacent nontumorous tissue were extracted for use in two batches of isobaric tags for relative and absolute quantitation experiments, which identified a total of 3220 proteins. A DAVID (database for annotation, visualization and integrated discovery) analysis of dysregulated proteins revealed that the three top-ranking biological processes were extracellular matrix organization, extracellular structure organization, and oxidation-reduction. Biological processes including response to organic substances, response to metal ions, and response to inorganic substances were highlighted by up-expressed proteins in bladder cancer. Seven differentially expressed proteins were selected as potential bladder cancer biomarkers for further verification. Immunohistochemical analyses showed significantly elevated levels of three proteins—SLC3A2, STMN1, and TAGLN2—in tumor cells compared with noncancerous bladder epithelial cells, and suggested that TAGLN2 could be a useful tumor tissue marker for diagnosis (AUC = 0.999) and evaluating lymph node metastasis in bladder cancer patients. ELISA results revealed significantly increased urinary levels of both STMN1 and TAGLN2 in bladder cancer subgroups compared with control groups. In comparisons with age-matched hernia urine specimens, urinary TAGLN2 in bladder cancer samples showed the largest fold change (7.13-fold), with an area-under-the-curve value of 0.70 (p < 0.001, n = 205). Overall, TAGLN2 showed the most significant overexpression in individual bladder cancer tissues and urine specimens, and thus represents a potential biomarker for noninvasive screening for bladder cancer. Our findings highlight the value of bladder tissue proteome in providing valuable information for future validation studies of potential biomarkers in urothelial carcinoma. Molecular & Cellular Proteomics 14: 10.1074/mcp.M115.051524, 2466–2478, 2015.

Bladder cancer is potentially lethal and is the most costly urologic malignancy to manage (1). Risk factors associated with bladder cancer include carcinogens in tobacco smoke and environmental and/or occupational exposure to chemical compounds. More than 380,000 new cases of bladder cancer are diagnosed worldwide, accounting for ~150,200 deaths each year (2, 3). Cystoscopic examination of the bladder coupled with voided urine cytology—the cytologic examination of cellular material present in the urine—is the “gold standard” for bladder cancer diagnosis. However, cystoscopy is a costly, invasive procedure that may require anesthetization of the patient. Moreover, voided urine cytology lacks sensitivity in diagnosing low-grade and early-stage tumors (4). Development of a convenient diagnostic and staging tool for the management and monitoring of bladder cancer is an ongoing worldwide effort (4, 5).

A number of studies seeking to discover bladder cancer biomarkers in urine have identified several abundant proteins as potential screening markers (6–9). We previously identified apolipoprotein A-I as a potential biomarker, showing a strong statistical association of this blood-associated protein with bladder cancer (7, 9). This linkage probably reflects that
Biomarker Discovery of Bladder Cancer by Tissue Proteomics

bleeding, secretion, and/or angiogenesis mechanisms are associated with bladder tumor. Biomarkers discovered directly through the urine proteome may have superior diagnostic sensitivity; however, they may not be sufficiently specific. Therefore, the application of urinary proteins that are abundant in plasma as cancer biomarkers is probably limited to certain subjects, such as high-risk patients or recurrence monitoring. One potential urinary biomarker discovered through profiling the urinary microparticle proteome of bladder cancer patients is tumor-associated calcium signal transducer 2 (TACSTD2) (10). Integration of the cancer cell secretome with tumor transcriptome analysis led to the discovery of urinary midkine (MDK) and hepatocyte growth factor activator inhibitor type 1 (HAI-1) as bladder cancer biomarkers (11). However, proteins that are abundant in body fluids may mask, and thus limit the detection, of tissue-leakage proteins present at low concentration.

Exploring the tissue proteome of clinical tissue specimens is an alternative, straightforward strategy for discovering potential biomarker candidates in human specimens. The expression level of such proteins in tissue potentially reflects the grade or stage of cancer progression and as such has the potential to serve as a prognostic marker. However, information about the bladder tissue proteome is limited. In a series of reports, Niu et al. studied the tissue proteome of bladder tumors using label-free shotgun proteomics, identifying 1753 differentially expressed proteins in four paired cancer and normal tissue specimens (12–16). Hundreds of differentially expressed proteins—all potential biomarker candidates—were used to study the carcinogenesis mechanism. To date, however, none of these candidates has been verified in individual tissue or urine specimens (14).

In the current study, we searched for potential bladder cancer biomarkers by profiling proteomic changes in tissue specimens of bladder tumors using a strategy combining laser capture microdissection (LCM)1, isobaric tags for relative and absolute quantitation labeling (iTRAQ), and linear trap quadrupole-Orbitrap tandem mass spectrometry (LTQ-Orbitrap MS/MS) analysis. Cancerous cells and adjacent non-tumorous cells were obtained by LCM from four pairs of surgically resected primary bladder cancer tissue specimens for two batches of four-plex iTRAQ experiments. A quantitative tissue proteome of the bladder tumor was generated and used to explore mechanisms associated with bladder cancer.

To access biomarker performance, we further verified the expression of selected dysregulated proteins in individual tissues and urine samples and correlated their expression with clinicopathological characteristics.

EXPERIMENTAL PROCEDURES

Clinical Specimens—Fresh-frozen specimens of surgically resected primary bladder tumor and adjacent nontumorous tissues were obtained from four patients (two females and two males). The specimens were immediately embedded in Tissue-Tek O.C.T. (Opti-Mem Cutting Temperature Compound (Sakura Finetek Inc., Torrance, CA) and stored at −80 °C until use. The tissue sections were stained with hematoxylin/eosin and evaluated by a pathologist before LCM experiments were performed. First morning urine specimens were collected from bladder cancer patients and hernia patients (used as controls for urinary protein biomarker verification studies) on the date of surgery. Clinical specimens were collected as previously described (6, 7). Briefly, urine samples were collected in the presence of a protease inhibitor mixture (one tablet/50 ml of urine; Roche, Mannheim, Germany) and sodium azide (1 mM) from hernia and bladder cancer patients. The collected samples were centrifuged at 5000 × g for 30 min at 4 °C within 5 h to remove cells and debris, and the clarified supernatants were stored at −80 °C before subsequent processing. Fresh-frozen tumor specimens for iTRAQ analysis were obtained from bladder cancer patient volunteers diagnosed at the Chang Gung Memorial Hospital, Taoyuan, Taiwan. The four frozen tissue specimens for iTRAQ experiments were collected between December 2008 and May 2010 and stored immediately at −80 °C. Formalin-fixed, paraffin-embedded tissue specimens were obtained from the tumor tissue bank of 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. All patients signed informed consent forms before collection of tissue and urine samples. Clinical information about patients participating in iTRAQ experiments (tissue biomarker discovery), immunohistochemistry (tissue biomarker verification), and enzyme-linked immunosorbent assay (ELISA) analysis (urinary biomarker verification) is presented in Supporting Information (Table III and supplemental Table S1). Sample statuses of low grade with early stage (LgEs), high grade with early stage (HgEs), and high grade with advanced stage (HgAs) are determined according to the TNM staging system.

LCM and Protein Extraction for LC-MS/MS Analysis—Proteins from tissue specimens were extracted by LCM within four months of sample collection using a modified sample preparation workflow described previously (17). Briefly, 16 μm cryosections were mounted onto membrane slides and fixed with 75% ethanol for 30 s. After washing with 25% ethanol for 60 s, they were placed in Mayer’s Decalcohol solution for 10–20 s, rinsed with 75% ethanol (10 s), dehydrated sequentially in 95 and 100% ethanol (30 s each), cleared twice in 100% xylene (5 min each), and thoroughly air-dried. LCM was performed using a Veritas Laser Capture Microdissection and Laser Cutting System (Arcturus, Mountain View, CA) as described by the manufacturer. Briefly, the tissue surrounding the selected area was cut using a UV laser, and the internal areas were irradiated with soft IR laser pulses to dissociate the cut sections from the membrane slides. Several selected areas were then adhered to a CapSure LCM Cap (Arcturus) and immediately transferred to a 0.5 ml microcentrifuge tube for protein extraction. All captured cells were dissolved in 100 μl of extraction buffer (0.5% RapiGest; Waters, Milford, MA) in 0.05 M triethylammonium bicarbonate buffer (TEAB; Sigma-Aldrich, Inc., St. Louis, MO) by vortexing at room temperature for 10 min. The concentration of protein in extracts was measured using the Bradford assay (Bio-Rad), and the protein profiles were further inspected by

1 The abbreviations used are: LCM, laser capture microdissection; CA2, carbonic anhydrase 2; HgEs, high grade and early stage; HgAs, high grade and advanced stage; iTRAQ, isobaric tags for relative and absolute quantitation; LC-MS/MS, liquid chromatography tandem mass spectrometry; LgEs, low grade and early stage; PGK1, phosphoglycerate kinase 1; RBC, red blood cell; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SFN, 14-3-3sigma; SLC3A2, 4F2 cell-surface antigen heavy chain; STMN1, stathmin; TPP, Trans-Proteomic Pipeline; TXN, thioredoxin; UTI, urinary tract infection; WBC, white blood cell.
sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining.

**iTRAQ Labeling and Fractionation of Labeled Peptides**—Proteins were extracted from 1.7 × 10^6 to 2.1 × 10^6 microdissected cells using 0.5% RapiGest SF (Waters) in 50 mM triethylammonium bicarbonate (TEAB), and digested with sequencing-grade modified trypsin (Promega, Madison, WI) at 37 °C overnight. For the discovery phase of the study, equal amounts of proteins extracted from tumor tissue and adjacent normal tissue from four bladder cancer patients were used for two batches of 4-plex iTRAQ experiments (Fig. 1). The desalted peptides were labeled using iTRAQ reagents (Applied Biosystems, Foster City, CA), according to the manufacturer’s instructions. In the first iTRAQ experiment, 53 μg of peptides from adjacent normal and tumor cells of patient 1 and adjacent normal and tumor cells of patient 2 were labeled with iTRAQ tags 114, 115, 116, and 117, respectively. In the second iTRAQ experiment, 43 μg of peptides from adjacent normal and tumor cells of patient 3 and adjacent normal and tumor cells of patient 4 were labeled with iTRAQ tags 114, 115, 116, and 117, respectively. For each experiment, the four iTRAQ-tagged peptide mixtures were then pooled and dried by vacuum centrifugation. The pooled mixtures of iTRAQ-labeled peptides were loaded onto a 4.6 × 150 mm Gemini C18 column (3-μm particles, 160-μm pore size; HPLC Phenomenex, Torrance, CA) and fractionated by basic reverse-phase chromatography using an increasing acetonitrile gradient containing 0.1% ammonium hydroxide at pH 10. Elution was monitored by measuring absorbance at 220 nm, and fractions were collected every 1 min. Effluents were collected and pooled into 40 fractions, based on signal intensity at OD_280nm. After vacuum drying, samples were ready for nano-ESI-LC-MS/MS analysis.

**LC-ESI MS/MS Analysis by LTQ-Orbitrap Pulsed-Q Dissociation**—Liquid chromatography-electrospray ionization (LC-ESI) MS/MS analysis of iTRAQ-labeled peptides was performed as previously described (7). Each separated peptide fraction was reconstituted in buffer A (0.1% formic acid in H_2O), and 2 μg of peptides from each fraction were loaded onto a trap column (Zorbax 300SB-C18, 0.3 × 5 mm; Agilent Technologies, Wilmington, DE) at a flow rate of 20 μl/min, and separated on a resolving 10-cm analytical BioBasic C_{18} PicoFrit column (inner diameter, 75 μm). Peptides were eluted at a flow rate of 0.25 μl/min across the analytical column with a linear gradient of 5–30% buffer B (0.1% formic acid in 99.9% acetonitrile) for 40 min, 30–45% buffer B for 5 min and 45–95% buffer B for 2 min, and then maintained in 95% buffer B for 4 min. The LC setup was coupled in-line to a LTQ-Orbitrap (Thermo Fisher, San Jose, CA) operating at Xcalibur 2.0 software (Thermo Fisher). Peptides were selected for MS/MS using the pulsed-Q dissociation (PQD) operating mode with a normalized collision energy setting of 27%; 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 scans were dynamically excluded for 180 s.

**MS Data Processing, Database Searching, and Protein Quantification**—MS data processing, database searches, and protein quantitation were performed as previously described (7). The resulting MS/MS spectra were searched against the European Bioinformatics Institute (http://www.ebi.ac.uk/) nonredundant International Protein Index (IPI) human sequence database (v3.55, Feb. 2009) containing 75,554 sequences and 31,556,873 residues using the Mascot engine (version 2.2.04; Matrix Science, London, UK) with the Mascot Daemon program (Matrix Science, version 2.2.0) to generate the peak list and DAT files. 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 digestes, oxidized methionine as a potential variable modification, and iTRAQ (N-terminal), iTRAQ (K), and methyl methanethiosulfonate (C) as fixed modifications. The charge states of peptides were set to +2 and +3. Detailed information about single-peptide-based protein identification is summarized in supplemental Fig. S7.

Protein identification and quantification were validated using the default setting of open source trans-proteomic pipeline (TPP) software (Version 4.0) for removal of redundancy as the final protein report. The MASCOT search resulted in a DAT file for each LC-MS/MS run. The MS raw data and DAT files containing peak list information for identified peptides were then processed and analyzed using TPP software, which includes PeptideProphet, a peptide probability score program that aids in the assignment of peptide MS spectra (18), and ProteinProphet, a program that assigns and groups peptides into a unique protein or a protein family if the peptide is shared among several isoforms (19). ProteinProphet allows filtering of large-scale data sets with assessment of predictable 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 0.9%. 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 peak 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 peptides 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/). The analytical reliability of iTRAQ coupling with Moscat search engine and TPP software has been shown in our previous work (7).

**Immunohistochemical Staining**—Immunohistochemical (IHC) staining of 5-μm-thick consecutive sections of formalin fixed, paraffin-embedded tissue specimens was performed as previously described (20). Antigens for carbonic anhydrase 2 (CA2), phosphoglycerate kinase 1 (PGK1), 14–3–3σ (SFN), 4F2 cell-surface antigen heavy chain (SLC3A2/CD98), transgelin-2 (TAGLN2), thioredoxin (TXN), and stathmin (STMN1) were retrieved by incubating with Bond Epitope Retrieval Solution 1 of the Bond-Max Automated Immunostainer (Vision BioSystems, Melbourne, Australia) at 100 °C for 20 min. Sections were stained at room temperature for 60 min on the Bond-Max system using a standard protocol. The following primary antibodies were used: rabbit polyclonal anti-CA2 (1:200; AB1828, Chemicon), goat polyclonal anti-PGK1 (1:100; sc_17943, Santa Cruz Biotechnology, Santa Cruz, TX); goat polyclonal anti-SFN (1:50; sc_7681, Santa Cruz Biotechnology); goat polyclonal anti-SLC3A2 (1:100; sc_7095, Santa Cruz Biotechnology); goat polyclonal anti-TAGLN2 (1:50; sc_51441, Santa Cruz Biotechnology), mouse monoclonal anti-TXN (1:50; sc_58440, Santa Cruz Biotechnology), and rabbit polyclonal anti-STMN1 (1:200; 569391, Merck). A polymer detection system (Bond Polymer Refine; Vision BioSystems) was used to reduce nonspecific staining. The IHC staining reaction was evaluated using a quantitative scoring method that is determined by two parameters: staining intensity and percentage of positive-stained cells. Staining intensity was scored on a scale of 0 to 3, where 0 represents no staining, and 1, 2, and 3 represent weak, moderate, and strong staining, respectively. The final score was obtained by multiplying the intensity (I) by the percentage of positive-stained cells (P). Using this system, we classified protein expression level in bladder cancer tissue and adjacent urothelium cells into three groups for each candidate cancer biomarker: low staining, 0–99 for TAGLN2 and SLC3A2, and 0–150 for STMN1; moderate staining, 100–199 for TAGLN2, 100–159 for SLC3A2, and 151–200 for STMN1; strong staining, ≥200 for TAGLN2, 160 for SLC3A2, and ≥201 for STMN1.
ELISA for Quantification of Urine Proteins—Urine specimens from age-matched patients with hernia, another urological disease, were selected as controls for biomarker verification in urine. The urinary concentrations of the four candidate proteins were measured using commercial ELISA kits for SLC3A2 (Cusabio, Wuhan, China), SFN (Cusabio, Wuhan, China) and STMN1 (USCN Life Sciences, Wuhan, China), and an ELISA developed in house for TAGLN2. Assays using commercial ELISAs were performed according to their respective manufacturer’s instructions using 100 μl raw urine specimens for each ELISA. For the TAGLN2 ELISA assay, white polystyrene 96-well microtiter plates (Corning Corp., Corning, NY) were coated with rabbit anti-TAGLN2 (10234–2–AP; Proteintech Group, Inc., Chicago, IL) antibodies by incubating each well with 100 μl of a 3500 ng/ml solution of antibody in PBS overnight at 4 °C. After washing, the plates were blocked by adding 200 μl of PBS containing 1% bovine serum albumin (BSA; Sigma) per well and incubating for 2 h at room temperature. Recombinant TAGLN2 protein (TP303473; OriGene Technologies, Inc., Rockville, MD) was used as a standard. Mouse anti-TAGLN2 (sc-373928; Santa Cruz Biotechnology) antibody, diluted 1:50 in PBS containing 1% BSA, was then applied and plates were incubated for an additional 1 h at room temperature. One hundred microliters of alkaline phosphatase-conjugated goat anti-mouse IgG (sc-2008; Santa Cruz Biotechnology), diluted 2000-fold in PBS containing 1% BSA, was added and plates were incubated for 40 min at room temperature. The substrate 4-methylumbelliferyl phosphate (Molecular Probes, Eugene, OR) was diluted to 100 μM with an alkaline phosphatase buffer:PBS mixture (1:3), and 100 μl was added to each well. The fluorescence was measured with a SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale, CA) using excitation and emission wavelengths of 355 and 460 nm, respectively.

Identification of Pathways Linked to Differentially Expressed Proteins Using DAVID Bioinformatics Resources—A network analysis of differentially expressed proteins was performed using the tools of the web-accessible DAVID 6.7 program (http://david.abcc.ncifcrf.gov/) (21–23). The relevant pathway maps were then prioritized based on their statistical significance (p < 0.001).

Statistical Analysis—The statistical SPSS software package (Version 12.0.1C; SPSS Inc., Chicago, IL) was used for all analyses of protein expression levels in individual samples determined by IHC and ELISA assays. Relationships between the concentrations of candidate biomarker proteins and different clinical parameters were analyzed using the nonparametric Mann-Whitney U test. Receiver operator characteristic (ROC) curve and area under the curve (AUC) analyses were applied to detect the optimal cutoff point that yielded the highest total accuracy with respect to discriminating different clinical classifications. Two-tailed p values ≤ 0.05 were considered significant. The optimal cut-off point for protein concentration in an ELISA was determined as the concentration that can yield the largest Youden’s index (J) in the receiver operating characteristic (ROC) curve, which is calculated as J = 1 − (false positive rate + false negative rate) = 1 − [(1 − sensitivity) + (1 − specificity)] = sensitivity + specificity − 1 (24). The Chi-square test was employed to identify significant clinicopathological characteristics in clinical tissue specimens of bladder cancer patients. Statistical significance was set at p < 0.05.

RESULTS

Identification and Quantification of Proteins in Four Individual Tissue Samples in Two Batches of iTRAQ Experiments—In the biomarker discovery phase, cells from four pairs of specimens of cancerous and adjacent nontumor tissue from surgically resected primary bladder cancer were obtained for use in two batches of iTRAQ experiments (Fig. 1C). Fig. 1A shows representative images of tumor and nontumor sections used for LCM. The proteins extracted from the four pairs of microdissected samples were examined by SDS-PAGE followed by silver staining (Fig. 1B). The peak area ratios of protein expression in tumor and nontumor cells (T/N ratio) of the same patient determined by iTRAQ reporter ions were used to select biomarker candidates. iTRAQ experiments 1 and 2 identified 2325 and 2477 proteins, respectively. Integration of the proteins identified in the two clinical sample sets yielded a total of 3217 unique bladder tissue proteins. Of these, 1585 (49.3%) were identified in both experimental runs. Protein/peptide identification and quantitation details for the two clinical sample sets are shown in Fig. 2. MS/MS spectra and corresponding fragment assignments for the single, distinct, peptide-based protein identifications are summarized in supplemental Fig. S7A and S7B. iTRAQ ratios of 1866 proteins and 2067 proteins were quantified in clinical sample sets 1 and 2, respectively; 1248 proteins (46.5%) were quantified in both sample sets. Among the total 2685 quantified proteins, those displaying a fold-difference ≥ 1.50 or ≤ 0.67 between a tumor subgroup and its adjacent normal subgroup based on at least one LIBRA peptide were defined as increased or decreased proteins, respectively. These quantified proteins were classified into the following four categories: (1) bladder cancer > nontumor: 131 proteins; (2) bladder cancer ≤ nontumor: 181 proteins; (3) not significantly different between bladder cancer and nontumor: 227 proteins; and (4) others: 2146 proteins. The definitions and numbers of quantified proteins in each subgroup are summarized in Table I. Protein identification and quantification results of the two iTRAQ experiments are detailed in supplemental Table S2A and S2B; up- and down-regulated proteins are listed in supplemental Table S3A and S3B.

Verifications of the Diagnostic/Prognostic Values of Biomarker Expressions in Tissue Specimens—On the basis of the iTRAQ results and relevance to human cancer, we selected seven candidate biomarkers—CA2, PGK1, SFN, SLC3A2, STMN1, TAGLN2, and TXN—which overexpressed in at least three of the four paired microdissected tissue specimens for further validation in individual tumor samples by IHC. Tumor tissue slides were scored for staining intensity and percentage of positive cells by a pathologist; The IHC scores for three candidates—SLC3A2, STMN1, and TAGLN2—were significantly higher in tumor cells than in noncancerous bladder epithelial cells (p < 0.001, n = 119–195; Table II, Fig. 3, and supplemental Fig. S1). Differences in the IHC expressions of the three proteins between tumor and adjacent normal cells of each individual patient using the dashed lines as well as all specimens are detailed in supplemental Fig. S1A–S1C and supplemental Table S7. The corresponding fold changes of average IHC scores were 3.9 for SLC3A2 (AUC = 0.937), 1.9 for STMN1 (AUC = 0.876), and 29.8 for TAGLN2 (AUC = 0.999). Owing to the inherent properties of bladder tumor tissues, adjacent noncancerous epithelium cells were found in
only 10–20% of the individual sections of paraffin-embedded tissue specimens. The percentage of cells staining positive for TAGLN2, which showed the largest fold-increase in IHC scores between tumor tissue cells and noncancerous cells \((p < 0.001\), Fig. 3C), was 100% (121/121) in tumor cell specimens and 25% (3/12) specimens with adjacent noncancerous cells within the same sections of paraffin-embedded IHC slides. TAGLN2 staining intensity was not detectable or weak in 95% (58/61) additional specimens of normal bladder tissue specimens, with a IHC score less than five in the individual sections \(n = 61\). In contrast, 86.8% (105/121) of positive-staining tumor specimens showed medium to strong TAGLN2 staining intensity by IHC. Additional 61 paraffin-embedded tissue slides of normal bladder further confirmed the lower IHC scores compared with tumor cells (Fig. 3A). A representative staining pattern for one paired tissue section and IHC scoring results of the three potential marker proteins are shown in Fig. 3A–3C. 

**Fig. 1.** A, LCM-dissected urothelial cells of bladder tumor tissue (T) and adjacent noncancerous (N) cells from four bladder cancer patients. Tissue cryosections were fixed and stained with hematoxylin/eosin (HE) for pathological analysis. Tissue specimens were stained with hematoxylin for use in LCM experiments. The region used for protein extraction is marked with red circles and arrows. B, SDS-PAGE analysis of proteins extracted from microdissected cells. C, Two batches of iTRAQ experiments were used to analyze microdissected bladder tumor and adjacent noncancerous tissue specimens from four bladder cancer patients. Peak area ratios of 115/114 (T1/N1 and T3/N3) and 117/116 (T2/N2 and T4/N4) reporter ions were used to indicate changes in expression between tumor and adjacent normal cells.

**Fig. 2.** Identification and quantification of proteins in two batches of iTRAQ experiments using four paired, microdissected bladder tumor specimens.

in IHC score, were 99.2% (120/121) and 38.5% (5/13) in tumor cells and adjacent noncancerous cells, respectively, within the same sections of paraffin-embedded IHC slides. SLC3A2 staining intensity was weak in all five specimens of positive-staining noncancerous cells, whereas 73.3% (88/121) of the positive-staining tumor specimens showed medium to strong SLC3A2 staining intensity by IHC. Additional 61 paraffin-embedded tissue slides of normal bladder further confirmed the lower IHC scores compared with tumor cells (Fig. 3A). A representative staining pattern for one paired tissue section and IHC scoring results of the three potential marker proteins are shown in Fig. 3A–3C. Supplemental Fig. S2A–S2D show Western blot of antibody validation data (full length) for SLC3A2, STMN1, TAGLN2, and SFN that used in immunohistochemistry. Peptide competition for TAGLN2 antibody is shown as supplemental Fig. S2E to confirm the specificity of antibodies used in this study.

The associations between overexpression of SLC3A2, STMN1, or TAGLN2 proteins and clinicopathological characteristics of bladder cancer patient tumor specimens were assessed using Chi-squared tests. All the three proteins showed a significant correlation with tumor grade, with higher IHC scores observed in higher-grade tumor slices \((p = 0.004 – 0.015)\). The IHC score for STMN1 were higher in large tumors (>2 cm) than small tumors. The IHC scores for SLC3A2 and TAGLN2 were higher in advanced TNM stage, defined ac-
Biomarker Discovery of Bladder Cancer by Tissue Proteomics

Table I
Summary of protein identification and quantification in the bladder tumor proteome of four individual patients in two batches of iTRAQ experiments

| Classification | Description | ITRAQ-1 (Patient 1 and 2) | ITRAQ-2 (patient 3 and 4) | Combination |
|----------------|-------------|---------------------------|---------------------------|-------------|
| Identified Proteins                                | 2325         | 2477                      | 3217                      |
| Quantified Proteins (QP)                           | 1866         | 2067                      | 2685                      |
| (I) cancer>normal (T/N > 1.5)                       | n = 4<sup>a</sup> | 88                        | 88                        | 6           | 131          |
|                                                          | n = 3<sup>b</sup> |                           |                           | 53          |
|                                                          | n = 2<sup>c</sup> |                           |                           | 72          |
| (II) cancer<normal (T/N < 0.67)                      | n = 4<sup>d</sup> | 110                       | 151                       | 13          | 181          |
|                                                          | n = 3<sup>e</sup> |                           |                           | 70          |
|                                                          | n = 2<sup>f</sup> |                           |                           | 98          |
| (III) No change                                       | 0.67<four T/N ratios<1.5 | 856                       | 581                       | 227          |
| (IV) Others                                            | (QP)-(I)-(II)-(III) | 815                       | 1247                      | 2146         |

<sup>a</sup> Proteins show increased expressions in all of the four patients.
<sup>b</sup> Proteins show increased expressions in any three of the four patients.
<sup>c</sup> Proteins show increased expressions in two patients and no identification or quantification results in the remaining two patients.
<sup>d</sup> Proteins show down expressions in all of the four patients.
<sup>e</sup> Proteins show down expressions in any three of the four patients.
<sup>f</sup> Proteins show down expressions in two patients and no identification or quantification results in the remaining two patients.

Table II
Details of biomarker discovery data and verification of seven selected protein candidates in tissue and urine specimens

| Protein name                       | GENE | T1/N1 | T2/N2 | T3/N3 | T4/N4 | IHC verification in tissue specimens (Cancer vs. normal) | Biomarker Discovery (Tissue) | Urine (Western blot) | Urine (ELISA) |
|------------------------------------|------|-------|-------|-------|-------|----------------------------------------------------------|-----------------------------|---------------------|-----------------|
| Carbonic anhydrase 2               | CA2  | 2.65  | 1.82  | 3.15  | 2.91  | no significant differences                               | grade discriminator         |                     |                 |
|                                    |      |       |       |       |       | (p = 0.008, AUC = 0.675, n = 84) stage discriminator    | (p = 0.027, AUC = 0.666, n = 84) |
| Phosphoglycerate kinase 1          | PGK1 | 0.94  | 2.35  | 2.61  | 1.83  | no significant differences                               | Diagnosis marker (p = 0.005, AUC = 0.675, n = 114) stage discriminator | (p = 0.010, AUC = 0.671, n = 84) |
|                                    |      |       |       |       |       | (p = 0.035, AUC = 0.648, n = 84) stage discriminator    | (p = 0.001, AUC = 0.675, n = 84) |
| Isoform 1 of 14–3-3 protein sigma  | SFN  | 1.89  | 2.07  | 8.94  | 0.83  | no significant difference, n = 174                     | N.D.                        |                     |                 |
| Isoform 2 of 4F2 cell-surface      | SLC3A2| 1.63  | 1.45  | 3.02  | 3.94  | p < 0.001, AUC = 0.937, n = 195                          | N.D.                        |                     |                 |
| antigen heavy chain                | (CD98)|      |       |       |       |                                                          |                              |                     |                 |
| Stathmin                           | STMN1| 1.96  | 2.50  | 0.80  | 8.04  | p < 0.001, AUC = 0.876, n = 119                          | Diagnosis marker (p = 0.001, AUC = 0.672, n = 152) stage discriminator | (p = 0.03, AUC = 0.631, n = 104) |
| Transgelin-2                       | TAGLN2| 2.88  | 1.57  | 1.23  | 1.95  | p < 0.001, AUC = 0.999, n = 194                          | Diagnosis marker (p < 0.001, AUC = 0.697, n = 205) stage discriminator | (p = 0.001, AUC = 0.712, n = 137) |
| Thioredoxin                         | TXN  | 1.55  | 1.82  | 2.39  | 0.83  | no significant differences                               | N.D.                        |                     |                 |
|                                    |      |       |       |       |       | (p = 0.001, AUC = 0.682, n = 137) stage discriminator   | (p = 0.001, AUC = 0.682, n = 137) |

According to the World Health Organization/International Society of Urological Pathology (24, 25). Specimens from patients with lymph node metastasis showed higher IHC scores for TAGLN2 than those without metastasis (p = 0.008), suggesting that TAGLN2 might be a useful molecular tumor marker for evaluating bladder cancer lymph node metastasis. Detailed statistical results of Chi regression analyses of correlations with clinicopathological characteristics are presented in Table III.

Verifications of the Diagnostic Performances of Biomarkers in Urine—Urine, which is in direct contact with bladder tissue, is a collection site for local drainage of tumor-derived proteins. Thus, the dysregulation of biomarker candidates in urine may signal the presence of a bladder tumor (10). Accordingly, we accessed the concentrations of the seven selected candidates in bladder cancer and nonmalignant urological disease patients (hernia) by Western blotting or ELISA, and then evaluated their correlation with disease severity. SFN and...
SLC3A2 proteins were not detectable in urine under the Western blotting and ELISA conditions we used for verification. Quantification of urinary protein concentration by Western blotting revealed that the average concentrations of CA2 and PGK1 were 1.4- to 3.3-fold and 2.1- to 4.5-fold higher, respectively, in individual samples from bladder cancer subgroups compared with those from the hernia subgroup (supplemental Fig. S3). Urinary concentrations of STMN1 and TAGLN2 were quantified by ELISA assays. For STMN1, ELISAs showed a 1.8- to 3.2-fold increase in three bladder cancer subgroups compared with urine samples from hernia patients \((n = 152; p = 0.001)\) with an AUC value of 0.67 (supplemental Fig. S3). Urinary TAGLN2 concentrations were strikingly elevated in all three bladder cancer subgroups, showing 3.25- to 16.87-fold increases compared with levels in urine from hernia patients \((n = 205, p < 0.001)\) (Fig. 4).

Among the candidates selected for verification in individual urine specimens, TAGLN2 showed the largest fold change \((7.13\text{-fold})\) in all bladder cancer patients compared with age-matched hernia controls, with an AUC of 0.70 using 333.0 ng/ml as the cut-off value \((p = 0.001; \text{Fig. 4A})\). In the sample cohort used for biomarker verification, the percentage of females was higher in bladder cancer than hernia control. To exclude the possible effect of androgen, the urinary TAGLN2 ELISA data was further analyzed using only male samples. supplemental Fig. S4 shows that the urinary TAGLN2 concentration remains significantly higher in male bladder cancer patients than male controls \((n = 97 \text{ versus } 65, p < 0.001, \text{AUC} = 0.70)\) suggesting that the higher urinary TAGLN2 levels in bladder cancer patients was not associated with sex. These four proteins—CA2, PGK1, STMN1, and TAGLN2—exhibited significant differences in concentration between early-stage and late-stage bladder cancer urine specimens, and thus could serve as stage discriminators. CA2, PGK1, and TAGLN2 were able to discriminate low-grade from high-grade cancer. Urinary concentrations of PGK1, STMN1, and TAGLN2 were significantly elevated in all bladder cancer patients compared with hernia patients (Table II). Up-expressions of tissue TAGLN2 and STMN1 in bladder tissue cells are one of the reasons that elevate the urinary TAGLN2 and STMN1 concentrations in bladder cancer urine specimens.

Some reported biomarker candidates may yield false-positive results in patients with hematuria or inflammation (25, 26) owing to the presence of blood cells in urine. The urine red blood cells (RBC) test, which is a common way to measure trace hematuria (HU) degree in urine, measures the number of red blood cells in a urine sample. The RBC number in urine higher than 20 cells/\mu l is defined as HU in clinic diagnosis. Before normalization of the TAGLN2 concentration by RBC values, we first performed the Spearman rank correlation analysis between urinary TAGLN2 and RBC values of control and bladder cancer urine specimens. Correlation analysis between urine RBC numbers and TAGLN2 levels \((n = 142)\), which showed that the two data were low-correlated \((\text{Spearman’s rank correlation coefficient } r = 0.376, \text{supplemental Fig. S6A})\). Numerous samples with the identical RBC values showed different values of TAGLN2 concentration. High TAGLN2 values were measured in many urine specimens with zero or low/normal RBC values (supplemental Fig. S6B). The elevated urine TAGLN2 levels could not be predicted by the RBC test in urine. Therefore, we think it is not appropriate currently to normalize the ELISA results directly using RBC values.

**Pathway Analysis by DAVID**—To interpret global changes in the tissue proteome associated with bladder cancer, we identified Gene Ontology (GO) biological processes that were
TABLE III
Correlations of SLC3A2, STMN1, and TAGLN2 protein expression (IHC scores) with clinicopathological characteristics in tissue specimens

| Characteristics       | SLC3A2 (CD-98) | STMN1 | TAGLN2 |
|-----------------------|----------------|-------|--------|
|                       | Score n        | Low (0–99) | Moderate (100–159) | High (160–300) | p value | Score n | Low (0–150) | Moderate (151–200) | High (201–300) | p value | Score n | Low (0–99) | Moderate (100–199) | High (200–300) | p value |
| Age                   |                |        |        |        |        |        |        |        |        |        |        |        |        |        |        |
| ≤65 years             | 39             | 12     | 21     | 6      | 0.243  | 15      | 5      | 6      | 4      | 1.000  | 39      | 3      | 31     | 5      | 0.615  |
| >65 years             | 75             | 14     | 52     | 9      |        | 32      | 11     | 12     | 9      |        | 75      | 10     | 54     | 11     |        |
| Sex                   |                |        |        |        |        |        |        |        |        |        |        |        |        |        |        |
| Male                  | 82             | 20     | 54     | 8      | 0.219  | 34      | 10     | 15     | 9      | 0.391  | 82      | 9      | 62     | 11     | 0.918  |
| Female                | 32             | 6      | 19     | 7      |        | 13      | 6      | 3      | 4      |        | 32      | 4      | 23     | 5      |        |
| Tumor size            |                |        |        |        |        |        |        |        |        |        |        |        |        |        |        |
| ≤2 cm                 | 51             | 16     | 29     | 6      | 0.170  | 18      | 11     | 4      | 3      | 0.018* | 51      | 7      | 39     | 5      | 0.350  |
| >2 cm                 | 56             | 9      | 38     | 9      |        | 26      | 5      | 13     | 8      |        | 56      | 6      | 39     | 11     |        |
| Histologic grade      |                |        |        |        |        |        |        |        |        |        |        |        |        |        |        |
| Low grade             | 32             | 13     | 18     | 1      | 0.007* | 15      | 10     | 4      | 1      | 0.004* | 32      | 8      | 21     | 3      | 0.015* |
| High grade            | 82             | 13     | 55     | 14     |        | 32      | 6      | 14     | 12     |        | 82      | 5      | 64     | 13     |        |
| Tumor status          |                |        |        |        |        |        |        |        |        |        |        |        |        |        |        |
| Ta+T1                 | 56             | 16     | 36     | 4      | 0.099  | 18      | 8      | 5      | 5      | 0.413  | 56      | 8      | 43     | 5      | 0.232  |
| ≥T2                   | 58             | 10     | 37     | 11     |        | 29      | 8      | 13     | 8      |        | 58      | 5      | 42     | 11     |        |
| Lymph node metastasis |                |        |        |        |        |        |        |        |        |        |        |        |        |        |        |
| N0                    | 99             | 21     | 67     | 11     | 0.091  | 37      | 13     | 14     | 10     | 1.000  | 99      | 12     | 77     | 10     | 0.008* |
| N1 + N2               | 15             | 5      | 6      | 4      |        | 10      | 3      | 4      | 3      |        | 15      | 1      | 8      | 6      |        |
| Distal metastasis     |                |        |        |        |        |        |        |        |        |        |        |        |        |        |        |
| M0                    | 108            | 23     | 70     | 15     | 0.214  | 42      | 15     | 15     | 12     | 0.610  | 108     | 12     | 82     | 14     | 0.309  |
| M1                    | 6              | 3      | 3      | 0      |        | 5       | 1      | 3      | 1      |        | 6       | 1      | 3      | 2      |        |
| TNM stage             |                |        |        |        |        |        |        |        |        |        |        |        |        |        |        |
| I + II                | 86             | 20     | 59     | 7      | 0.020* | 29      | 10     | 10     | 9      | 0.756  | 86      | 11     | 68     | 7      | 0.006* |
| III + IV              | 28             | 6      | 14     | 8      |        | 18      | 6      | 8      | 4      |        | 28      | 2      | 17     | 9      |        |
dysregulated by analyzing the proteins whose levels were significantly different between bladder cancer cells and adjacent noncancerous cells using the online DAVID bioinformatic website (21–23). Statistically significant GO biological processes \( p < 0.0001 \) associated with bladder cancer and the associated proteins are listed in supplemental Table S4. These analyses revealed that five biological processes were significantly associated with the 131 up-regulated proteins in bladder tumors \( p < 0.0001 \), including response to organic substances, response to metal ions, and response to inorganic substances. Among the 181 down-regulated proteins, oxidation-reduction, extracellular matrix organization, and organic acid catabolic process were the top three ranking associated biological processes. After integration of up- and down-regulated proteins, the top three ranking biological processes were extracellular matrix organization, extracellular structure organization, and oxidation-reduction, with \( p \) values of \( 1.61 \times 10^{-10}, 2.93 \times 10^{-9}, \) and \( 9.11 \times 10^{-9} \), respectively. The DAVID analysis also indicated a major involvement of dysregulated proteins in KEGG pathways related to amino acid metabolism. The pathways associated with dysregulated proteins are detailed in supplemental Table S5.

**DISCUSSION**

**Comparison of Tissue and Urine Proteomes in Bladder Cancer**—In this work, we used LCM to ensure the purity of cancer and normal cells for iTRAQ experiments. To understand the presence in urine of bladder tissue proteins associated with
Although this bladder cancer bladder tissue proteins with 1939 normal urinary proteins, downloaded from the Sys-Bodyfluid database compiled by Li et al. (27). This comparison revealed that CA2, PGK1, SFN, and TXN had been identified in the normal urine proteome (supplemental Fig. S5). For this category of proteins, we would expect to see an elevation in their urinary concentrations in bladder cancer patients compared with controls if the proteins were secreted from tissue to urine. However, the presence of these proteins (CA2 and PGK1) in the normal urine proteome could increase the background concentration, decreasing the fold change compared with urine samples from bladder cancer patients. It would also suggest that these urine proteins originate from multiple sources in the body circulation. We found that the fold changes in CA2 and PGK1 in bladder cancer urine were relatively small; thus, their use as diagnosis biomarkers would be problematic. In the current study, SFN and SLC3A2 were not detectable by Western blotting and ELISA in urine.

STMN1, TAGLN2, and SLC3A2, were not identified in the normal urine proteome of the Sys-Bodyfluid database, probably owing to their low concentration in normal urine. In the current study, we found that urinary STMN1 and TAGLN2 levels were significantly different between individual cancerous and noncancerous samples, and were able to discriminate bladder cancer from control. Notably, comparison of the results obtained here with the proteomic data in our previous work, which used iTRAQ to screen differentially expressed proteins in pooled samples of bladder cancer urine (supplemental Table S6), showed a significant increase in TAGLN2 in bladder cancer urine specimens (supplemental Table S6B), although this protein was not selected as a candidate for verification at the time. Consistent with this previous observation and the quantitative tissue proteome data presented here, we verified that TAGLN2 levels were significantly elevated in tissue specimens (by IHC) and urine (by ELISA) in the current study. Moreover, a proteomic analysis of condition mediums of U1 and U4 bladder cancer cell lines showed that both STMN1 and TAGLN2 are detectable in the bladder cancer secretome (28). Our previous work showed that TAGLN2 protein concentrations were increased ~12-fold in the urinary microparticle proteome of bladder cancer patients compared with controls (10). Taken together, these results suggest that up-regulated TAGLN2 in bladder tumor cells may be secreted into urine through microparticles, which in turn causes the higher concentration of urinary TAGLN2. Therefore, it is conceivable that secretion of STMN1 and TAGLN2 from tumor cells into urine may cause the increased urinary levels of both proteins. The clinical utility of urinary STMN1 and TAGLN2 as potential diagnostic biomarkers will require further validation in a larger number of specimens.

The Biology of Target Proteins in Tumorigenesis—Three proteins—TAGLN2, STMN1, and SL3CA2—were significantly up-regulated (p < 0.001) in tumor tissue cells compared with noncancerous cells in individual tissue sections. Among these proteins, TAGLN2 showed the greatest overexpression in bladder cancer tissue and urine specimens. Recently, Yoshino and colleagues reported that miR-1/miR-133a microRNA clusters may function as tumor suppressors in bladder cancer through repression of TAGLN2, showing that miR-1/miR-133a transfection or TAGLN2 knockdown decreased bladder cancer cell viability, inhibited cell proliferation, and induced apoptosis (29). They also observed a positive correlation between TAGLN2 expression and tumor grade in clinical bladder cancer tissue specimens. Conversely, down-regulation of miR-1 and miR-133a in human renal cell carcinoma resulted in up-regulation of the oncogenic TAGLN2 (30). Overexpression of TAGLN2 has been reported to serve as a potential biomarker for some cancer types, including colorectal cancer (31), hepatocellular carcinoma (32), and breast cancer (33, 34). To our knowledge, the current study is the first study to report that urinary TAGLN2 can be used as a noninvasive biomarker for bladder cancer diagnosis and grade/stage discrimination.

It has been reported that Stathmin, a p53-target gene (35), is overexpressed in many cancers and is associated with poor prognosis (36–39). STMN1 expression is increased by down-regulation of miR-193b, resulting in enhanced migration and proliferation of tumor cells in melanoma (40). A three-protein panel composed of PPARG, STMN1, and CAV2 has been shown to be associated with time to recurrence of bladder tumor (41). Bhagirath et al. reported that the serum and urinary concentrations of STMN1 were higher in urothelial carcinoma of the bladder than in controls (36). These observations, taken together with the results of our current tissue proteome study, again support the idea that measuring dysregulated tissue proteins in human bodily fluids is a feasible strategy for discovering potentially useful noninvasive biomarkers.

SLC3A2 (also termed CD98), an integrin-associated protein that mediates integrin-dependent signals, has been shown to promote tumorigenesis (42, 43). Abnormal expression of SLC3A2 mRNA or protein has been previously confirmed in gastric cancers (44), renal cell cancer (45), and colon cancer (46). The clinical histology data presented here establish the first association of SLC3A2 with bladder cancer. As part of our evaluation of SLC3A2 as urinary a biomarker, we sought to detect SLC3A2 protein in bladder cancer urine specimens using a commercially available ELISA. However, SLC3A2 was not detectable, probably because of the low concentration in urine, although the suitability of the assay for urine has not been established. The concentration distribution of SLC3A2 protein in urine and differences in the concentration between bladder cancer and control patients is currently not clear; these issues will require further study using a mass spectrometry-based approach or other assays.

Important Pathways—The most significant bladder cancer-associated biological process determined based on dysregu-
lated proteins identified here was extracellular matrix organization ($p = 1.61 \times 10^{-10}$; supplemental Table S4). Of the dysregulated proteins linked to this pathway, 11 were up-regulated and six were down-regulated. Collagen type I is transcribed from two separate genes, COL1A1 and COL1A2, both of which are methylated in association with decreased collagen expression in several human cancer cells (47, 48), and bladder cancer tissues (49). Our data also showed that COL1A1, COL1A2, and COL18A1 proteins were down-regulated in bladder tumor cells compared with adjacent normal cells of bladder cancer patients.

It has been reported that both the genetic factors and exposure to organic and inorganic substance, particularly metal carcinogens (50–54), are risk factors for bladder cancer (55, 56). Bladder cancer appears to be caused by exposure to harmful substances that lead to abnormal changes in bladder cells over the course of many years. Interestingly in this latter context, a number of up-regulated bladder tumor proteins identified in the current study are associated with biological processes including response to organic substances, response to metal ions, and response to inorganic substances. These proteins included epidermal growth factor receptor (EGFR), cytochrome P450 1A1 (CYP1A1), γ-glutamyl hydrolase (GGH), and CA2. Crossover between EGFR and androgen receptor pathways plays an important role in the progression of bladder cancer (57, 58). Studies by Wang and colleagues have shown a significant positive correlation between CYP1A1 genetic polymorphisms and an increased risk of bladder cancer among Asians and Europeans (59–61). Urinary GGH has been proposed as a urinary marker of bladder cancer for outcome prediction after chemotherapy (62). Little information is available about the association of CA2 with bladder cancer. Our iTRAQ data suggested such an association, showing that CA2 was up-regulated in tumor cells of the four paired LCM samples. However, these differences failed to reach significance in subsequent IHC verification experiments using the small sample size ($n = 14$). Further study will be required to confirm the relationship between CA2 expression levels and bladder cancer.

The proteomic study described here, incorporating LCM with the iTRAQ technique, generated accurate quantitative proteomic data from cancerous and adjacent normal cells, increasing the known bladder tumor proteome (to 3217 proteins) and identifying candidate biomarkers. In addition to providing valuable information for future validation studies of potential biomarkers for urothelial carcinoma, our study also revealed that exposure to toxic substances may mediate the up-regulation of some proteins described above and contribute to the increased risk of bladder tumor development.

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[**This article contains supplemental Figs. S1 to S7 and Tables S1 to S7.**]

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Biomarker Discovery of Bladder Cancer by Tissue Proteomics

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