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Evidence of Abnormal Tyrosine Phosphorylated Proteins in the Urine of Patients With Bladder Cancer: The Road Toward a New Diagnostic Tool?

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Abstract

Purpose

Since changes in protein phosphorylation are a common feature of cancer cells, we analyzed phosphoproteins in the tissue and urine of patients with bladder cancer and assessed the diagnostic relevance of abnormally phosphorylated proteins as tumor markers.

Materials and Methods

Enrolled in this study were 66 patients and 82 healthy volunteers. From the first 14 patients with bladder cancer we obtained samples of malignant and normal bladder tissue. All patients and volunteers provided a urine sample. Protein extracts of tissue specimens were separated by 2-dimensional gel electrophoresis for comparative analysis of neoplastic and normal tissue. Phosphoproteins were studied by Western blot and characterized by mass spectrometry. Urine samples were analyzed by 1-dimensional gel electrophoresis. Phosphoproteins were measured by affinity dot blotting.

Results

Profound changes in the pattern of protein tyrosine phosphorylation were consistently, reproducibly observed in bladder cancer tissues. A total of 24 phosphorylated proteins were differentially expressed in cancer tissue and identified by mass spectrometry. Phosphoproteins were fairly stable in urine samples, leading to accumulation. Urinary tyrosine phosphoproteins showed the most remarkable changes in patients with cancer with an approximately 5-fold increase compared to levels in healthy controls.

Conclusions

To our knowledge we investigated for the first time the diagnostic potential of tissue and urinary tyrosine phosphoproteins for bladder carcinoma. Results indicate that phosphorylated proteins may represent a new, valuable class of urinary biomarkers for bladder cancer.

Key Words: urinary bladder; carcinoma, transitional cell; tumor markers, biological; phosphoproteins; diagnosis

Abbreviations and Acronyms

1-DE, 1-dimensional gel electrophoresis;

2-DE, 2-dimensional gel electrophoresis;

SDS, sodium dodecyl sulfate;

TCC, transitional cell carcinoma

THE great interest in urinary markers of bladder TCC is proved by the numerous studies published each year that propose new, noninvasive tests. However, the large number of proposed tests and studies done in an effort to discover new markers also suggests that the problem is far from solved.

A noninvasive test for TCC is urgently needed. Cystoscopy, which continues to be the gold standard diagnostic method, is highly accurate, sensitive and specific. However, cystoscopy is also an uncomfortable procedure. Urine cytology is noninvasive but has a high rate of false-negative findings, especially in low grade TCC cases, underscoring the limit of this method.[1, 2 and 3]

A noninvasive urine test would be especially useful in the followup of patients with superficial TCC, in whom a noninvasive test could significantly decrease the need for cystoscopy with ensuing benefits for patient comfort and decreased followup costs, and in population groups at risk for TCC, such as heavy tobacco smokers and individuals with significant occupational exposure.[4, 5 and 6] Various traditional molecular biology and genetic studies, and more recently many studies that applied innovative techniques, such as epigenetic (DNA methylation analysis) and proteomic evaluations, have been done in the quest for urinary markers of TCC.[7 and 8] To date various new urine bladder markers have become available. However, most of these markers tend to be less specific than cytology and yield more false-positive results.[9] Only a few groups have simultaneously analyzed cancer tissue and urine to verify potential cancer markers in urine and test their true value.[10 and 11] Vrooman and Witjes provided evidence that most proteomic based markers have yet to be validated for diagnostic use.[12]

Since the discovery of changes in protein phosphorylation as initial events in cancer growth, and since the development of proteomic techniques, analysis of the phosphoproteome has been attracting ever increasing interest and a number of proteins that are abnormally phosphorylated in cancer have been reported. Protein kinases, which are enzymes responsible for protein phosphorylation, are involved in malignancy.[13 and 14] Particularly greater than 50% of tyrosine kinases[13] are implicated in human cancer and can harbor mutations that lead to cancer development.[14 and 15]

We identified abnormally tyrosine phosphorylated proteins in neoplastic tissue, notably in the urine of patients with TCC. Thus, increased tyrosine phosphorylated protein levels in urine may be a novel class of highly specific bladder cancer biomarkers.

Materials and Methods

Tissue and Urine Collection and Storage

From April 2007 to December 2009 patients with suspected TCC were enrolled in this study before undergoing transurethral resection of the bladder or radical cystectomy. Those with a histological diagnosis different from bladder TCC or with previous TCC were subsequently excluded from analysis. The study was approved by the local research ethical committee and done according to Helsinki Declaration principles. All participants in the protocol signed a declaration of informed consent.

Samples of bladder cancer tissue and macroscopically normal tissue distant from the bladder cancer were obtained from the first 14 patients with TCC (table 1). Tissue specimens were obtained during transurethral resection of the bladder by cold cup biopsy or by cold section of the bladder immediately after removal during radical cystectomy. The amount collected was about 5 to 10 mg of malignant and normal tissues. Specimens were snap frozen in dry ice within 10 minutes of excision and proteins were extracted as previously described.[16] A total of 59 male (89%) and 7 female patients (11%) with a mean ± SD age of 72.13 ± 11.78 years who had TCC, and 37 male (45%) and 45 female (55%) healthy volunteers with a mean age of 50.08 ± 15.73 years provided urine specimens from the second micturition of the morning, which is the same void that is usually required for urine cytology. Samples were collected in sterile tubes and processed within 2 hours of sampling.

Table 1.

TCC pathological stage and grade

	No. Pts (%)
T stage:	
Ta	27 (41)
T1	14 (21)
T2	12 (18)
T3–T4	9 (14)
Cis	4 (6)
N stage:	
N0	61 (92)
N+	5 (8)
Grade:	
G1	16 (24)
G2	14 (21)
G3	32 (49)

Two-Dimensional Gel Electrophoresis

Proteins from tissue were resolved by 2-DE, performed as previously described.[16] For the second dimension 12% SDS-polyacrylamide gels were run. Preparative gels were stained with colloidal Coomassie stain and analytical gels were stained with silver nitrate.[16]

Western Blotting

Proteins resolved by electrophoresis were blotted onto Immobilon™-P polyvinylidene fluoride membranes (Millipore). After blotting membranes were incubated with anti-phosphotyrosine antibody PY 20 or anti-phosphoserine antibody PSR-45 (Sigma-Aldrich®). After incubation with anti-mouse secondary antibodies conjugated with IRDye® 800CW infrared fluorescent dyes excitable at 800 nm immunoreactivity was detected by the Odyssey® Infrared Imaging System.

Mass Spectrometry Protein Identification

We identified proteins by in-gel digestion and peptide mass fingerprinting using matrix assisted laser desorption ionization-time of flight mass spectrometry with a TofSpec SE (MicroMass, Wythenshawe, United Kingdom) according to manufacturer standard protocols using α-cyano-4-hydroxycinnamic acid (Sigma®). The 25 most intense masses were used for database searches against the Swiss-Prot 57.6 database (Swiss Institute of Bioinformatics, Geneva, Switzerland). The parameters used in the searches were taxa Homo sapiens, trypsin digest, 1 missed cleavage by trypsin, carbamidomethylation of cysteine as fixed modification, methionine oxidation and tyrosine phosphorylation (only differential phosphoproteomic analysis) as variable modifications with a maximum error allowed of 100 ppm.

Urine Processing

Urine samples were centrifuged at 700 × gravity for 20 minutes at 10C. Supernatant was enriched in phosphoproteins with Cu-immobilized metal ion affinity chromatography according to manufacturer standard protocols for chelating HP columns (GE Healthcare Bio-Sciences, Piscataway, New Jersey). To verify phosphoprotein stability urine samples were incubated at 37C for 30 minutes, and 2, 4 and 6 hours at physiological pH 5.5 and pH 8.0, adjusted by adding NaOH. For Western blot phosphoproteins were precipitated with acetone 50% (volume per volume) for 20 minutes in wet ice and centrifuged at 2,700 × gravity for 5 minutes at 4C.

One-Dimensional Gel Electrophoresis

Proteins from tissue and precipitated proteins from urine supernatant were solubilized with Laemmli buffer.[17] Equal amounts of proteins were resolved on 10% SDS-polyacrylamide gels. Western blot was done as described.

Affinity Dot Blotting

Anti-phosphotyrosine antibodies PY 20 (Santa Cruz Biotechnology, Santa Cruz, California) (1 μl, diluted 1:5,000) was spotted onto Hybond™ ECL nitrocellulose membrane and allowed to dry. The nitrocellulose membrane was incubated with eluted phosphoproteins from 5 ml of the urine sample and then probed with anti-phosphotyrosine antibodies PY 20 (Sigma-Aldrich). After incubation with anti-mouse secondary antibodies conjugated with IRDye 800CW infrared fluorescent dye excitable at 800 nm immunoreactivity was detected by the Odyssey Infrared Imaging System.

Analysis

Image

We performed 2-DE stained analytical gels and Western blot image analysis using PDQuest™, version 7.2. We performed 1-DE Western blot and affinity dot blot quantifications by densitometric analysis with Odyssey V3.0.

Statistical

Image analysis data were used as values of protein expression or phosphorylation. For 2-DE only proteins that were over expressed or over phosphorylated at least 2.5-fold in cancer compared to healthy specimens were considered and only protein identifications with a Mascot score of greater than 55 were considered significant. To verify the significance of variations in expression and phosphorylation of proteins in bladder cancer vs normal specimens, and between different disease stages and grades we used Student's t test with statistical significance considered at p ≤0.05. For affinity dot blotting we also performed ROC curve analysis to assess sensitivity and specificity at various urine phosphotyrosine cutoffs to detect TCC. The power of model predicted values to discriminate between positive and negative cases was quantified by the ROC AUC using MedCalc, version 11.3.3.

Results

Bladder Tissue Proteomic and Phosphoproteomic Analysis

To detect over expressed and tyrosine/serine over phosphorylated proteins 14 paired tissue specimens of bladder carcinoma and corresponding urothelium distant from the neoplastic lesion were comparatively analyzed. Figure 1 shows representative 2-DE gel images of normal and neoplastic bladder tissues. Protein separation was efficient, allowing the study of greater than 1,000 protein spots. Figure 1, B shows proteins with statistically significant over expression in bladder cancer with respect to normal tissue.

Figure 1.

Representative 2-DE proteome analysis of bladder tissue. *A*, normal. *B*, TCC. Circles indicate position of identified protein spots (table 2) over expressed in TCC vs normal tissue. *MW*, molecular weight. *pI*, isoelectric point.

Table 2 shows the mass spectrometry identification of all over expressed proteins sorted by frequency, that is the number of tumor samples in which the protein was over expressed at least 2.5-fold with respect to that in normal tissue. Five proteins, corresponding to spots 7, 2, 3, 4 and 5, were found in a high percentage of neoplastic specimens. Notably these proteins were characterized by low expression. No correlation was found between cancer stage and specific protein over expression in our patient cohort. Table 2 also shows the average fold increase, expressed as the cancer-to-normal ratio, and the p value for each over expressed protein.

Table 2.

Proteins over expressed in cancer vs normal bladder tissue samples on matrix assisted laser desorption ionization-time of flight mass spectrometry

No. Paired Samples▯	Spot No.†	Accession No.	Protein Name	No. Matched (% coverage)‡	Mascot Score	Ca/Normal	Ca vs Normal p Value
13	7	Q13268	Dehydrogenase/reductase SDR family member 2	6(24)	74	Greater than 100	<0.001
11	2	Q6GMP2	α-Enolase	11(31)	135	Greater than 100	<0.001
11	3	Q9P1Y4	Keratin, type I cytoskeletal 19	9(23)	98	Greater than 100	<0.001
11	4	Q9BV79	Trans-2-enoyl-CoA reductase, mitochondrial	5(12)	58	Greater than 100	<0.001
11	5	P22626	Heterogeneous nuclear ribonucleoproteins A2/B1	7(23)	74	Greater than 100	<0.001
11	1	P04040	Catalase	6(14)	56	2.8	<0.01
10	9	P15531	Nucleoside diphosphate kinase A	9(64)	142	4.1	<0.05
10	10	P22392	Nucleoside diphosphate kinase B	7(57)	96	4.1	<0.05
9	6	Q00688	FK506-binding protein 3	6(24)	66	3.0	<0.01
9	8	Q9H963	Zinc finger protein 702	4(41)	63	3.1	<0.05

▯

Total 14 paired samples.

†

Figure 1, *B*.

‡

Total 25 most intense matches.

Figure 2 shows representative phosphoproteome patterns in normal and malignant bladder tissues. Comparative analysis done by 2-DE Western blot on the 14 tumor tissue samples and their paired controls showed no significant differences in serine phosphorylated proteins (fig. 2, *A* and *B*). In contrast, the number and intensity of tyrosine phosphorylated proteins was markedly increased in cancer tissues (fig. 2, *C* and *D*). All bladder cancer tissue samples showed fairly reproducible tyrosine phosphoproteomic patterns, leading to the identification of a number of significantly over phosphorylated proteins.

Figure 2.

Representative 2-DE Western blot phosphoproteome analysis shows phosphorylation patterns in bladder tissue. *A*, serine phosphorylation in normal tissue. *B*, serine phosphorylation in TCC. *C*, tyrosine phosphorylation in normal tissue. *D*, tyrosine phosphorylation pattern in TCC. *D*, circles indicate position of identified protein spots that were more tyrosine phosphorylated in TCC than in normal tissue. *MW*, molecular weight. *pI*, isoelectric point.

To prove the specificity of the anti-phosphotyrosine and anti-phosphoserine Western blots the proteins were enzymatically dephosphorylated by treatment with alkaline phosphatase before separation. This resulted in the complete loss of signal, indicating the high specificity of phosphoprotein detection (data not shown). We used mass spectrometry to identify all tyrosine over phosphorylated proteins in TCC specimens, sorted by the number of tumor samples in which protein was over expressed at least 2.5-fold with respect to normal tissue. We determined the average fold increase, expressed as the cancer-to-normal ratio, and the p value for each over expressed protein. Many over phosphorylated proteins were apparently present only in tumor specimens but not in normal tissue, expressed as a cancer-to-normal ratio of greater than 100. Only cytokeratin 19 and heterogeneous nuclear ribonucleoproteins A2/B1 were over expressed as well as tyrosine over phosphorylated (table 2).

Tyrosine Phosphorylated Proteins in Urine

Analysis

Since tyrosine phosphorylated proteins appear to be potential TCC markers, we analyzed urine samples from patients with bladder cancer and healthy donors for phosphoproteins. To enrich for phosphoproteins we applied a Cu-immobilized metal ion affinity chromatography protocol to all urine samples analyzed. The 1-DE Western blots with anti-phosphoserine antibodies revealed serine-phosphorylated proteins in urine but no significant differences were found between tumor and control samples (fig. 3,*A*). Conversely anti-phosphotyrosine Western blots identified tyrosine phosphorylated proteins in urine and clearly demonstrated their increase in number and intensity in all urine samples from patients with TCC. Figure 3, *B* shows representative electrophoretic patterns. Figure 3, *A* and *B* shows anti-phosphoserine and anti-phosphotyrosine Western blots of normal urothelium and bladder carcinoma in comparison to the urinary phosphoprotein patterns. Some tyrosine phosphoprotein bands showed the same mobility in urine and tissue samples, suggesting a correspondence between urine and bladder tissue phosphoproteins. Figure 3, *C* shows the overall quantification of tyrosine phosphorylated proteins by Western blot in urine samples from 14 healthy controls and 14 patients with bladder cancer. We observed an approximately 4.5-fold average increase of phosphorylated protein levels in the urine of patients with TCC compared to healthy controls. Notably we observed no overlapping results between the individual values measured in the 2 groups.

Figure 3.

Representative 1-DE Western blot urine phosphoproteome analysis. *A*, protein serine phosphorylation pattern in urine samples of controls (*lane a*), patients with TCC (*lane b*), normal bladder tissue (*lane c*) and TCC tissue (*lane d*). *B*, protein tyrosine phosphorylation pattern in urine samples of controls (*lane a*), patients with TCC (*lane b*), and normal (*lane c*) and neoplastic (*lane d*) bladder tissue. *C*, densitometric analysis shows mean ± SD tyrosine phosphorylated proteins in urine samples of 14 controls (open bar) and 14 patients with TCC (gray bar). Asterisks indicate p <0.001.

Stability

Tyrosine phosphorylated proteins in urine at pH 5.5 were substantially stable. Conversely phosphoproteins were rapidly de-phosphorylated at pH 8.0, indicating that low pH is a major determinant of phosphoprotein stability in urine (fig. 4).
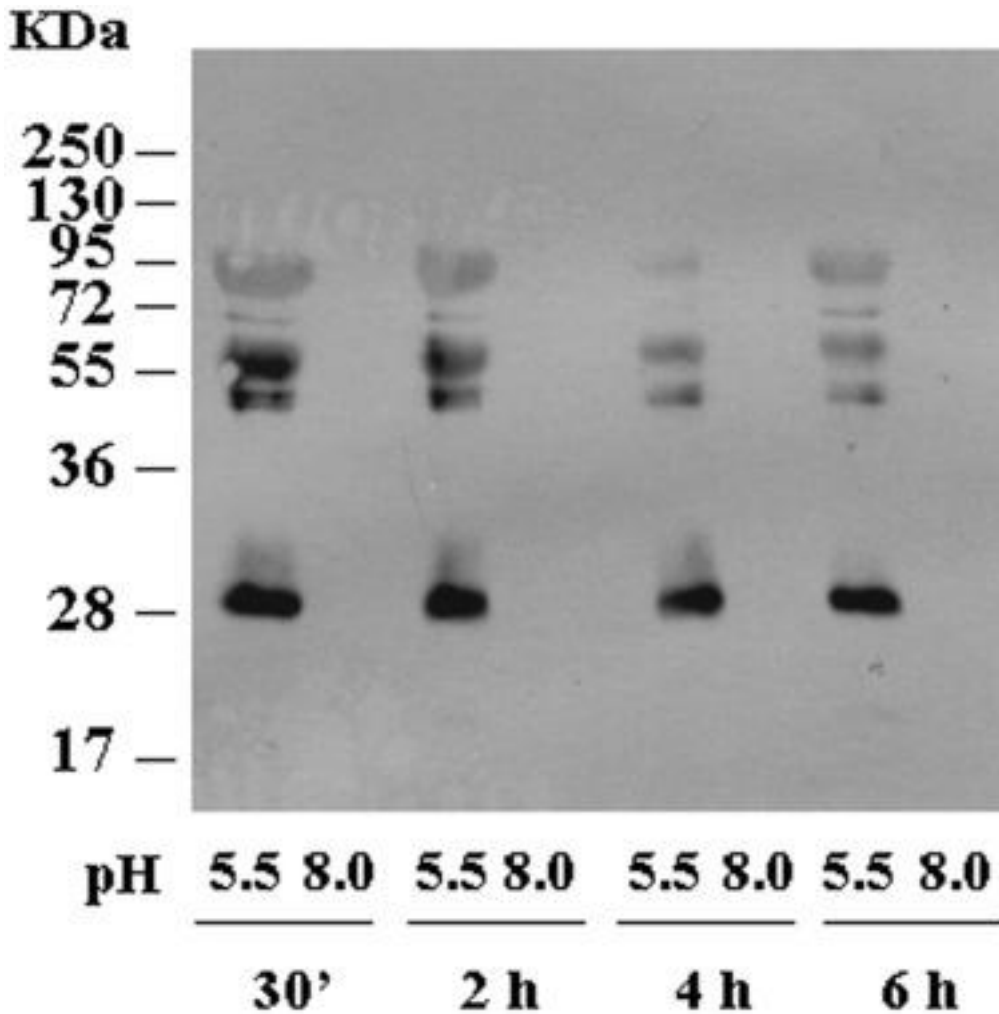
Figure 4.

Protein tyrosine phosphorylation stability in urine samples of patients with TCC incubated for 30 minutes, and 2, 4 and 6 hours (*h*) at 37C, and pH 5.5 and 8.0. Urine supernatant precipitated proteins (60 μg) were separated on 10% SDS-polyacrylamide gel and 1-DE gels were analyzed by Western blot.

Affinity Dot Blotting

To investigate the diagnostic role of tyrosine phosphorylated protein levels in the urine of patients with TCC we analyzed a larger number of urine samples using a high throughput method. Figure 5,*A* shows the levels of tyrosine phosphorylated proteins assessed by affinity dot blot in urine samples from 68 healthy controls and 52 patients with bladder cancer. Results showed a significant difference between tyrosine phosphorylated protein levels in patients with bladder cancer vs healthy controls (mean 2.93 ± 1.06 vs 0.61 ± 0.35), corresponding to an approximately 5-fold increase in patient urine. We performed ROC curve analysis (fig. 5, *B*). When the entire phosphotyrosine level range was considered, the ROC AUC was 0.997 (95% CI 0.963–1.000, p <0.0001). Table 3 shows the diagnostic validity criteria, such as sensitivity and specificity at different decision limits of the ROC curve. High cancer stages and grades were not associated with higher phosphotyrosine levels than in low cancer stages and grades, indicating that this analysis maintained its high sensitivity and specificity for low grade TCC (data not shown).
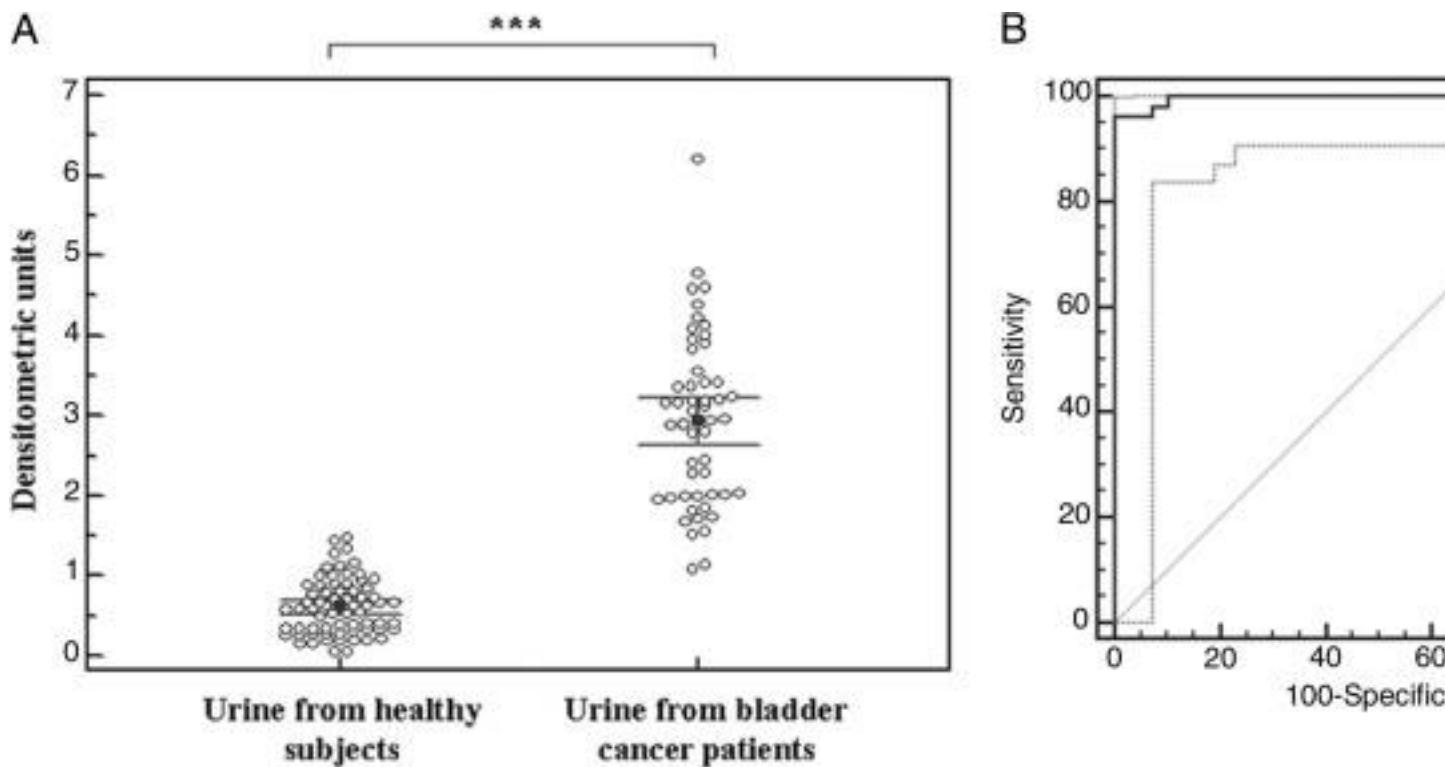
Figure 5.

Tyrosine phosphorylated protein levels in urine samples of 68 controls and 52 patients with TCC were analyzed by affinity dot blotting. *A*, tyrosine phosphorylated protein levels. Asterisks indicate p <0.001. *B*, ROC curve of total urine phosphotyrosine.

Table 3.

Sensitivity and specificity at different urine phosphotyrosine cutoffs

Phosphotyrosine Cutoff (densitometric U)	% Sensitivity (95% CI)	% Specificity (95% CI)
0.04orGreater	100.00 (93.20–100.00)	0.00 (0.00–5.30)
Greaterthan1.02	100.00 (93.20–100.00)	89.71 (79.90-95.80)
Greaterthan1.07	98.08 (89.70–100.00)	89.71 (79.90–95.80)
Greaterthan1.12	98.08 (89.70–100.00)	92.65 (83.70–97.60)
Greaterthan1.15	96.15 (86.80–99.50)	92.65 (83.70–97.60)
Greaterthan1.48	96.15 (86.80–99.50)	100.00 (94.70–100.00)
Greaterthan6.21	0.00 (0.00–6.80)	100.00 (94.70–100.00)

Discussion

Although there is strong evidence that cancer cells or their components are shed in the urine of patients with TCC, the value of available diagnostic markers is limited.[9] Previous reports have often focused on separate analyses of neoplastic tissues and urine, and

only a few groups have examined cancer tissue markers and their true value as urinary markers in parallel.[10 and 11] To our knowledge no previous study has been done to search for phosphoprotein changes in bladder cancer.

To specifically gear our study toward the identification of relevant urinary markers we performed a comprehensive proteomic and phosphoproteomic study of neoplastic tissue and paired control tissue. We also validated the most promising markers by evaluating their presence in urine samples. To select diagnostic markers with the greatest potential for use at all disease stages we studied a cohort of 14 patients with TCC encompassing all cancer stages. Proteomic analysis of the neoplastic tissue covered more than 1,000 proteins and revealed that 5 were apparently expressed only in neoplastic tissue while another 5 were over expressed. All identified proteins were previously associated with different carcinomas.[18, 19, 20, 21 and 22] However, to our knowledge dehydrogenase/reductase SDR family member 2, trans-2-enoyl-CoA reductase mitochondrial, FK506-binding protein 3 and zinc finger protein 702 have not been previously associated with cancer forms and may represent specific urinary cancer markers.

In any case notably all of the proteins we found selectively expressed in cancer showed low abundance and were close to the detection limit. Under such circumstances we could not accurately measure the cancer-to-normal tissue ratio. We also could not exclude the presence of additional proteins that may show high selectivity for cancer tissue but have been below our detection limit. Furthermore, as a result of the large dilution of a TCC marker in urine, the measurement of low abundance diagnostic targets may hinder their practical application.

In contrast to protein expression patterns, the tyrosine phosphoproteome revealed striking differences between cancer and normal tissue. The tyrosine phosphorylated protein changes in neoplastic tissue were not only more numerous than the observed protein expression changes but much more intense and easily recognizable. Bearing in mind that our samples were from patients representing all disease stages, the observed reproducibility of the phosphoprotein changes was somewhat unexpected and represented strong motivation to pursue the search for tyrosine phosphorylated protein markers in urine. In contrast, changes in serine phosphorylated proteins were not consistently selective for neoplastic tissue.

To our knowledge we found and investigated for the first time the diagnostic potential role of tyrosine phosphorylated protein levels in the urine of 66 patients with TCC. Mirroring the situation in cancer bearing tissues, we noted that the urine samples of patients with TCC showed clear alterations in tyrosine phosphorylated protein patterns. Also, tyrosine phosphorylated proteins were significantly over phosphorylated in TCC urine samples, strongly suggesting their potential application as a new class of urinary biomarkers for bladder carcinoma. The stability of urinary phosphoproteins at physiological pH 5.5 is the crucial factor for their accumulation and detection, as evidenced by their instability at higher pH. Tyrosine phosphorylation is a key regulatory mechanism that controls many cellular events, such as growth and proliferation,[23] which may explain the marked differences between patients with TCC and healthy controls. New high throughput and cost-effective analytical methods are now required to extend this study to a higher number of samples. For this purpose we are currently characterizing the tyrosine phosphorylation sites of urinary phosphoproteins to develop specific antibodies.

Conclusions

To our knowledge we report for the first time the presence of profoundly modified protein tyrosine phosphorylation patterns in the neoplastic tissue and urine samples of patients with TCC. Quantitative measurement of the level of tyrosine phosphorylated proteins in urine revealed that it effectively discriminated between controls and patients with cancer. Our results indicate that tyrosine phosphorylated proteins represent a new class of bladder cancer markers.

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