

*Advances in Brief***Detection of Bladder Cancer Using a Novel Nuclear Matrix Protein, BLCA-4<sup>1</sup>**

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**Abstract**

We have identified previously six nuclear matrix proteins (NMPs) that are bladder cancer specific. In this study, we analyzed the expression of one of these proteins, BLCA-4, in bladder tumors and normal bladder tissue. We also examined the appearance of BLCA-4 in the urine as a biomarker for bladder cancer. BLCA-4 was isolated from nuclear matrix preparations of bladder tumors, and its peptide sequence was determined. The antibodies generated against the resulting BLCA-4 peptides were then used to detect its presence in immunoblots and in urine samples by immunoassay. We analyzed tissue samples of bladder tumor and normal donor bladders and urine obtained from 51 normal individuals and 54 patients with pathologically confirmed bladder cancer. The BLCA-4 peptide sequences do not resemble any known human protein sequences. On immunoblot analysis, BLCA-4 expression was detectable in tumor and normal tissues from patients with bladder cancer but not in any of the normal bladder tissue obtained from organ donors. Using a prospectively determined cutoff level of 13 A (absorbance) units/ $\mu$ g protein, all 51 normal individuals tested were negative for BLCA-4 expression, whereas 53 of 55 samples from patients with bladder cancer were positive. These results suggest that BLCA-4 is present throughout the bladder in both the tumor and morphologically normal areas in bladder cancer patients. BLCA-4 is a very sensitive (96.4%) and specific (100%) marker for bladder cancer.

BLCA-4 is a bladder cancer-specific marker that can be detected using a urine-based assay and can be used in the diagnosis of bladder cancer.

**Introduction**

In the United States, bladder cancer is the fourth most common cancer in men and the eighth most common cancer in women. An estimated 53,200 cases of bladder cancer will be diagnosed in 2000, and it will account for >12,200 deaths (1). Several important risk factors have been identified for bladder cancer including cigarette smoking, exposure to chemicals such as aniline dyes, benzidine compounds, aromatic amines, “slow acetylators” metabolic phenotypes (2), and the presence of chronic inflammation or infection of the bladder (3). Histologically, >90% of bladder cancers are transitional cell carcinomas; squamous cancers and adenocarcinomas constitute 5–6 and 1%, respectively (4).

Currently, the only available method for bladder cancer detection is morphological examination of cytology samples or cystoscopic biopsies. Voided urine cytology is accurate for high-grade lesions; however, a significant proportion of bladder tumors (25–45%) are low grade or well differentiated and escape detection upon examination of exfoliated cells. The sensitivity of urine cytology is higher for carcinoma *in situ* and poorly differentiated tumors, while being fairly low for low-grade or well-differentiated tumors (5). Repeating the study can increase the sensitivity of cytology; however, this is a costly and time-consuming practice for both the patient and physician. When bladder cancer is detected early at a localized stage, the 5-year survival rate is 94%. Disease that has spread regionally or distantly lowers survival to 49 and 6%, respectively (6). Development of a sensitive diagnostic test that could specifically detect bladder carcinoma would significantly facilitate patient management and allow earlier treatment of this disease.

One characteristic that is common to all cancer cells is abnormal nuclear shape and the presence of abnormal nucleoli. These alterations are so prevalent in cancer cells that they are commonly used as a pathological marker of transformation. Nuclear shape reflects the internal nuclear structure and processes and is determined, at least in part, by the nuclear matrix (7). The nuclear matrix plays a central role in the regulation of important cellular processes such as DNA replication and transcription (8). The nuclear matrix is the framework or scaffolding of the nucleus and consists of the peripheral lamins and pore complexes, an internal ribonucleic protein network, and residual nucleoli (9). The nuclear matrix consists of ~10% of the nuclear proteins and is virtually devoid of lipids, DNA, and histones (10).

Although all cell types and physiological states share the

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majority of known NMPs,<sup>3</sup> some NMPs appear to be unique to certain cell types or states. We have demonstrated previously that the protein composition of the nuclear matrix is tissue specific and can serve as a “fingerprint” of each cell and/or tissue type (11). Mitogenic stimulation and the induction of differentiation alter the composition of nuclear matrix proteins and structure (12, 13). Differences in NMP composition are also found in a number of human tumors including prostate (14, 15), renal (16), breast (17), colon (18), cervical (19), and head and neck (20, 21). These data provide a strong rationale for our investigation into the differences in NMP composition that might be discerned in normal *versus* malignant bladder tissue. Previously, another urine-based test for NMPs has been used for the detection of bladder cancer with variable results. The NMP22 test (Matritech, Inc., Newton, MA) measures urinary levels of the nuclear mitotic apparatus protein (NUMA), and high levels of this protein have been demonstrated to be present in patients with recurrent bladder cancer (22). The protein detected by the NMP22 test can be found in the nuclear matrix of most cell types and is not specific to bladder cancer.

In an earlier study, we examined normal and tumor bladder tissue samples from 24 patients undergoing surgery for bladder cancer at the University of Pittsburgh Medical Center (23). All patients had transitional cell carcinoma. The NMP compositions of the 24 tumors and their corresponding normal tissue were then analyzed, using a computer-based gel analysis system. The nuclear matrix compositions of all tumors were found to be different from the nuclear matrix compositions of the matched normal tissue from the same bladder. We identified six proteins (BLCA-1 to BLCA-6) that were present in all of the tumors and were absent in the adjacent normal tissue and three proteins that were found in all of the normal bladder tissue samples and were missing in the tumor samples. Tumors used in these studies were of varying nuclear grades. These differences appear to be unique to bladder cancer in that the molecular weights and isoelectric points of the proteins do not appear to correspond to those of the nuclear matrix proteins reported previously to be different in prostate, renal, breast, head and neck, or colon cancers. We also examined a number of normal tissue sites and did not find expression of these proteins. We have developed a technique for sequencing proteins isolated from spots in two-dimensional gels and used this technique to sequence the three most abundantly expressed bladder cancer-associated NMPs, BLCA-1, BLCA-4, and BLCA-6. The resulting peptide sequence data were used to raise antibodies against these peptides as well as to synthesize degenerate oligonucleotides that could be used to screen a cDNA library to obtain the entire cDNA sequences. The focus of our current report is to characterize the expression patterns of one bladder cancer-associated protein in particular, BLCA-4.

## Materials and Methods

Bladder tumor tissue and adjacent “normal” tissue was used for immunoblot analysis. These tissues were obtained from 12 patients undergoing radical cystectomy at the University of

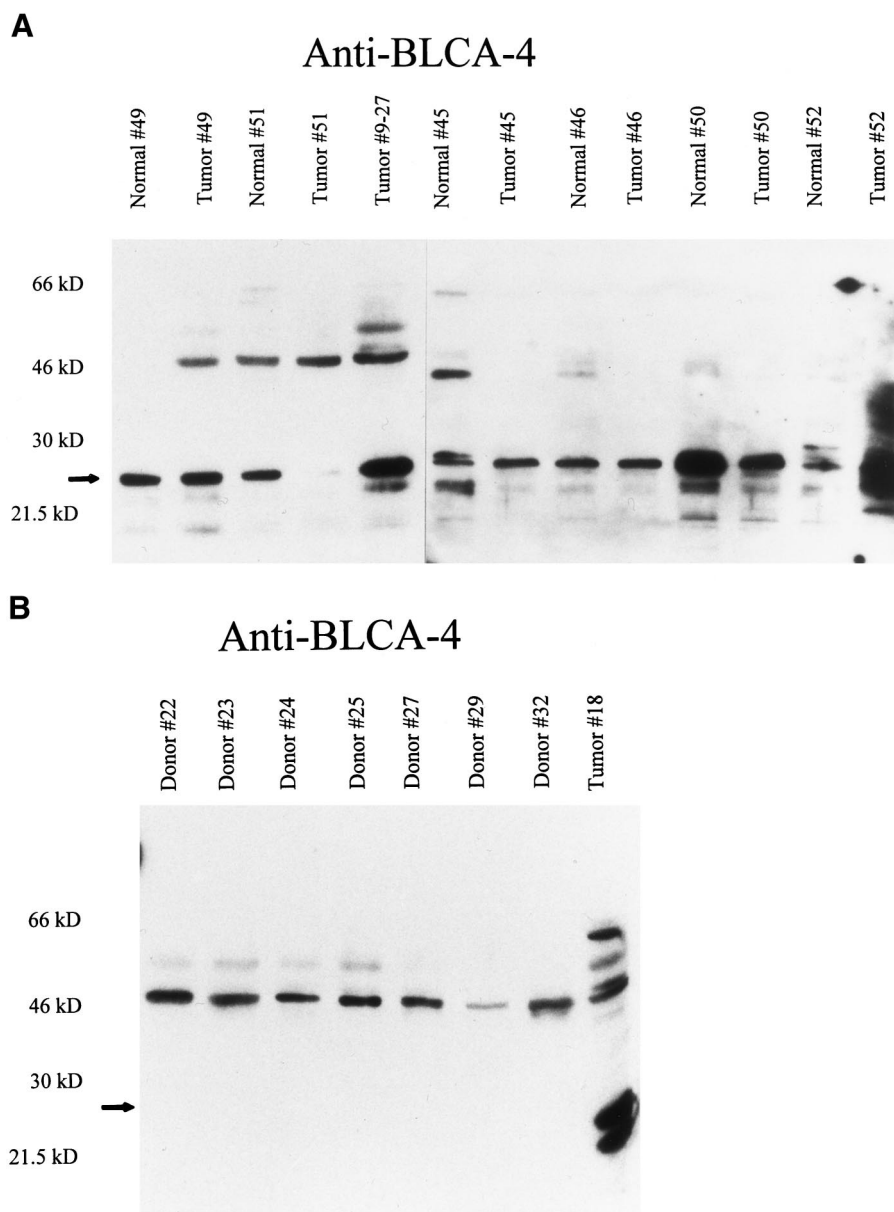
Pittsburgh Medical Center. Truly normal bladder tissue was obtained from 11 unaffected individuals who were organ donors and died of causes unrelated to bladder cancer. The patients ranged in age from 43 to 79 years with a mean age of 61.8 years ( $\pm 11$  years). Thirty-eight % of the sample population was female. In the immunoblot studies, most of the patients had invasive transitional cell carcinomas with grades of 3–4. Age ranges in the organ donor population were 19–67 years with a mean age of 36.9 years ( $\pm 19$  years). To control for age differences, only samples from individuals  $>40$  years of age were used in these studies. All samples were confirmed by the pathologist as containing either relatively pure samples of tumor or normal cells. Nuclear matrix proteins were extracted for immunoblot analysis using the method of Fey and Penman as modified by Getzenberg *et al.* (14). The approximate amount of each sample used was 1 g, from which about 300–500  $\mu\text{g}$  of NMPs were extracted and used for performing multiple immunoblot analyses.

A standard protocol was followed in the production of antibodies raised against one of the BLCA-4 peptides. Using the peptide sequence derived from sequencing, the corresponding spots from high-resolution, two-dimensional gels, peptides were designed from which to raise antibodies. The peptides produced were modified slightly to include the addition of terminal cysteines for coupling purposes, along with several amino acids for spacing to increase immunoreactivity. The sequences were verified through mass spectroscopy and conjugated to keyhole limpet hemocyanin. The sequence that we used for the BLCA-4 antibody was acetyl-EISQLNAGAhxC-amide. The resulting antigen was suspended in saline and emulsified by mixing with an equal volume of Freund’s adjuvant. Two New Zealand White rabbits (3–9 months of age) received injections of the peptide in three to four s.c. dorsal sites four times over a 3-month period. The animals were bled from the auricular artery, and the serum was collected from three-production bleeds. Antibodies were produced by Quality Controlled Biochemicals (Hopkinton, MA).

Immunoblot analysis was performed according to standard established protocols. Twenty  $\mu\text{g}$  of each sample of extracted NMP suspended in PBS were loaded and separated by 10% SDS-PAGE. Ten  $\mu\text{l}$  of Rainbow markers (Amersham Life Sciences, Arlington Heights, IL) were also loaded. Proteins were then transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA), and the membrane was incubated overnight in 10% nonfat dry milk in Tris-buffered saline (TBS) with 0.1% Tween at 4°C. The membrane was then washed with TBS and 0.1% Tween, followed by a 1-h incubation with a 1:500 dilution of anti-BLCA-4 antiserum and 10% nonfat dry milk at 4°C. The membrane was further washed with TBS and 0.1% Tween and incubated for 1 h in 1:20,000 dilution of goat antirabbit IgG secondary antibody conjugated with horseradish peroxidase (Pierce Chemical Co., Rockford, IL). The membrane was washed again with TBS and 0.1% Tween, and proteins were detected by a chemiluminescence reaction using the ECL immunoblot kit (Amersham Life Sciences).

The detectability of BLCA-4 using the anti-BLCA-4 antibody was assessed by using serial dilutions of BSA-conjugated anti-BLCA-4 antiserum against known concentrations of BLCA-4 peptide coated into the wells of a 96-well plate and by performing an immunoassay. Urine samples from patients with

<sup>3</sup> The abbreviations used are: NMP, nuclear matrix protein; CI, confidence interval.



*Fig. 1* Examples of immunoblot analyses of BLCA-4 in bladder tissues. *A*, the anti-BLCA-4 antibody (42280) was used to detect the protein in bladder cancer samples and normal adjacent bladder tissues from patients undergoing cystectomies for bladder cancer. As indicated by the *arrows*, the BLCA-4 antibody is not able to differentiate between tumor and adjacent pathologically normal tissues and normal bladder samples from organ donors that do not have bladder cancer (*B*). In individuals without bladder cancer, the antibody did not react with any of the samples that we examined. In addition to the band corresponding to BLCA-4, we found a band of  $M_r \sim 50,000$ , which is a background band often seen with anti-NMP antibodies.

pathologically confirmed bladder cancer, along with normal controls, were collected and tested with an ELISA that we developed. The urine samples were precipitated in cold, absolute ethanol (1:3) on ice (4°C) for 30 min. The samples were then centrifuged at  $1877 \times g$  at 4°C for 30 min. The pellet was suspended in appropriate volumes with PBS (1×). The protein concentration of each of the precipitated urine samples was then determined using the Coomassie Plus assay (Pierce Chemical Co., Rockford, IL) and read at an absorbance of 500 nm. For coating the high-binding 96-well plates, 50  $\mu$ l each of sample were added to each well. Rabbit IgG was used as a positive control in these studies. The Rabbit IgG (Southern Biotechnology Associates, Inc., Birmingham, AL) was diluted at 1:2000 with room temperature TBS (25 mM Tris/HCl, 0.15 M NaCl) and

incubated overnight at room temperature. The plates were rinsed three times with deionized water and blocked with 300  $\mu$ l of 1% BSA blocking buffer in TBS (1% BSA, 1% nonfat powdered milk, and 0.05% Tween 20) for 30 min at room temperature. The washes were repeated before treating the wells with antibody. The primary BLCA-4 anti-peptide antibody was added to the sample wells (50  $\mu$ l/well), which was diluted at 1:10 in 2.5% BSA Blocking buffer in TBS (2.5% BSA, 2.5% nonfat powdered milk, and 0.05% Tween 20). Negative controls consisting of normal rabbit serum (preimmune) diluted in 2.5% BSA Blocking buffer at 1:10 were added to another column and incubated 2 h at room temperature. After the incubation, the washes were repeated. The secondary antibody was goat-anti-rabbit (Southern Biotechnology Associates, Inc., Birmingham,

Table 1 Summary of BLCA-4 immunoblot data (percentage of samples with positive staining)

	Individuals with bladder cancer		Controls (organ donors)
	Tumor tissue <sup>a</sup>	"Normal" tissue <sup>b</sup>	Normal tissue
No. positive	9/12 (75%)	12/12 (100%)	0/11 (0%)
95% CI	42.8%–94.5%	73.5%–100%	0%–28.5%

<sup>a</sup> Compared with controls,  $P < 4 \times 10^{-4}$  (exact test).

<sup>b</sup> Compared with controls,  $P < 10^{-6}$  (exact test).

AL) diluted at 1:1000 with 2.5% BSA Blocking buffer. To each well, except for the blank wells on the template, the secondary antibody (50  $\mu$ l/well) was added and incubated for 2 h at room temperature. The plates were washed, wrapped in plastic wrap, and stored at 4°C overnight. To all of the wells, including the blank, 75  $\mu$ l of room temperature *p*-nitrophenyl phosphate substrate (3 mM *p*-nitrophenyl phosphate, 0.05 M sodium carbonate, and 0.05 mM magnesium chloride) were added, incubated for 1 h, and read at 405 nm. To assess variability of the results between assays, three precipitated normal control samples and one tumor urine sample were run, along with each batch of precipitated tumor urine samples. Each sample was run multiple times. To determine the range of detectability of BLCA-4 in the urine and variability within each assay, we conducted immunoassays with four samples from patients with bladder tumors and one normal control using serial dilutions of sample over a range of dilutions from 1:10 to 1:160. The initial precipitated protein concentrations of these five samples (four tumor and one normal) ranged from 0.397 to 1.958  $\mu$ g/ $\mu$ l. All studies were performed according to Institutional Review Board-approved protocols. Statistical analysis was performed using a two-sided Wilcoxon signed rank sum test for quantitative determinations and the Fisher exact two-sided test for qualitative determinations.

## Results

Using protein spots isolated from two-dimensional gels, we obtained two peptide sequences corresponding to distinct regions of one of the bladder cancer-specific NMPs, BLCA-4. The first peptide has a 75% homology with a number of nonvertebrate proteins including the Staph epidermis epiB protein and several yeast proteins. The second peptide of BLCA-4 has 75% homology with *Arabidopsis thaliana* ERECTA and lower levels of homology with other proteins in the BLAST database. No homology was found between these two BLCA-4 peptide sequences and any known or characterized human proteins.

Using antibodies raised against a single BLCA-4 peptide, we performed immunoblotting analysis of bladder cancer samples and normal adjacent bladder tissues from patients undergoing cystectomies for bladder cancer (Fig. 1A). As indicated by the arrows, the BLCA-4 protein is expressed in both tumor and normal tissue in patients with bladder cancer. This positive staining in the morphologically defined normal tissues was evident regardless of the area of the bladder from which it was taken and the proximity to the tumor site. The protein was found even in areas remote from the visible tumor. In these samples, we also find a band of  $M_r \sim 50,000$  that is considered a back-

ground band that we often see with anti-NMP antibodies. This approximately  $M_r \sim 50,000$  protein appears to have an affinity for immunoglobulins, regardless of their origin.

The expression of BLCA-4 was then analyzed by immunoblot analysis of normal bladder tissue samples from organ donors free of bladder cancer. As demonstrated in Fig. 1B, the antibody did not react with any of the samples that we examined. In addition to the organ donor bladder samples, we included a single tumor sample obtained from a patient who underwent cystectomy for the treatment of bladder cancer. This single sample produced positive reactivity in a band corresponding with the expected size for BLCA-4. In addition to the band corresponding to BLCA-4, the background band described above was often evident. Thus, as opposed to the morphologically "normal" areas in the bladders of individuals with bladder cancer, the presumably truly normal samples from individuals without bladder cancer do not react with the antibody. As described in Table 1, we have recently been able to expand these series of studies. To date, we have found positive BLCA-4 staining in 100% (12 of 12) of the "normal" tissues in the bladders of patients with bladder cancer. In the corresponding tumor samples, 75% (9 of 12) showed BLCA-4 expression. The remaining three tumor samples with no detectable levels at standard exposure did show low levels of expression upon further exposure of the blots. When the bladders from unaffected individuals were examined, expression was not found in any of the 11 samples examined, even when these blots were overexposed.

The possible confounding effect of age differences was of concern. The patients with bladder cancer ranged in age from 43 to 79 years, with a mean age of 62 years. The organ donor population ranged in age from 19 to 67 years, with a mean age of 37 years. To achieve approximate comparability in age distribution, donors <40 years were excluded from analysis. The age restriction was chosen blinded to the BLCA-4 outcomes. The association between BLCA-4 and disease states was highly significant ( $P < 4 \times 10^{-4}$ , Fisher exact two-sided test).

Recently, using a urine-based immunoassay, we have been successful in detecting BLCA-4 in 55 urine samples from 54 patients with histologically proven bladder cancer and 51 normal volunteers. After testing various dilutions of anti-BLCA-4 antibody against known concentrations of the BLCA-4 peptide, the dilution yielding optimal detectability was 1:10, and this dilution was adopted for all future immunoassays. A "normal" cutoff value of 13 A units/ $\mu$ g of total urinary protein was established after an assay of the first three samples. This cutoff value was then used in a prospective manner and applied to all of the samples subsequently assayed. The variability of results between assays was determined by comparing BLCA-4 values obtained by repeated testing of one tumor urine sample and three normal control samples. Variation in BLCA-4 levels obtained was an average of 1.87 A units/ $\mu$ g protein (range, 1.3 to 2.8) for the tumor sample and 1.07 A units/ $\mu$ g protein (range, 0.1–2) for the normal control samples. The range over which the BLCA-4 levels varied with serial dilution (up to 160-fold) are shown in Fig. 2. We have also been able to detect peptide that has been added to unprecipitated urine samples (in 0.5 and 0.15 mg/ml concentrations) with adequate recovery over a wide range of dilutions from 1:1 to  $1:2.4 \times 10^8$  (data not shown). The

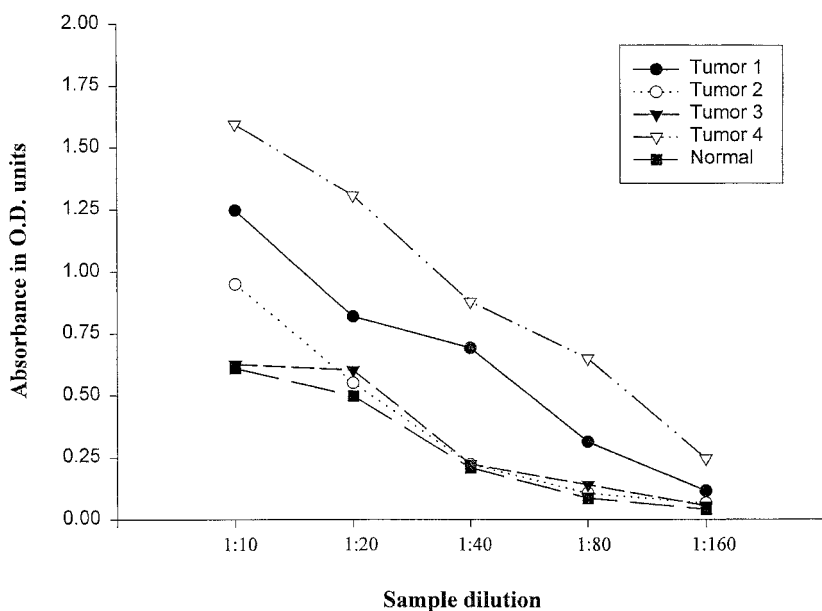
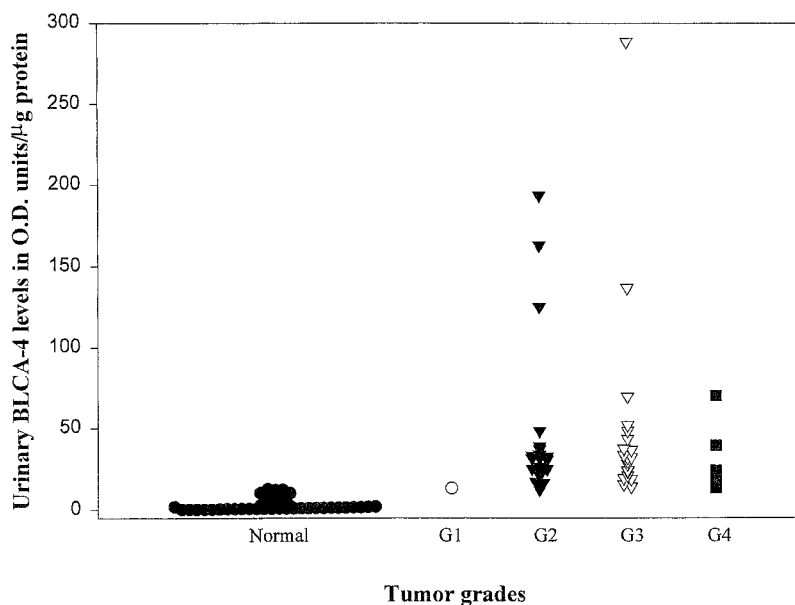


Fig. 2 Absorbance obtained in an immunoassay for BLCA-4 using serial dilutions of four urine samples from patients with bladder tumor and one normal control volunteer. The sample dilutions ranged from 1:1 to 1:160. The same dilution of anti-BLCA-4 antiserum (1:10) was used against all sample dilutions.

Fig. 3 Distribution of urinary BLCA-4 levels according to grade of tumor. Of the 54 patients whose urine samples were analyzed, 1 was grade 1, 22 were grade 2, 20 were grade 3, and 6 were grade 4. Four patients had no stated grade because they had carcinoma in situ, which is not assigned any grade. The grade was unavailable in one patient.

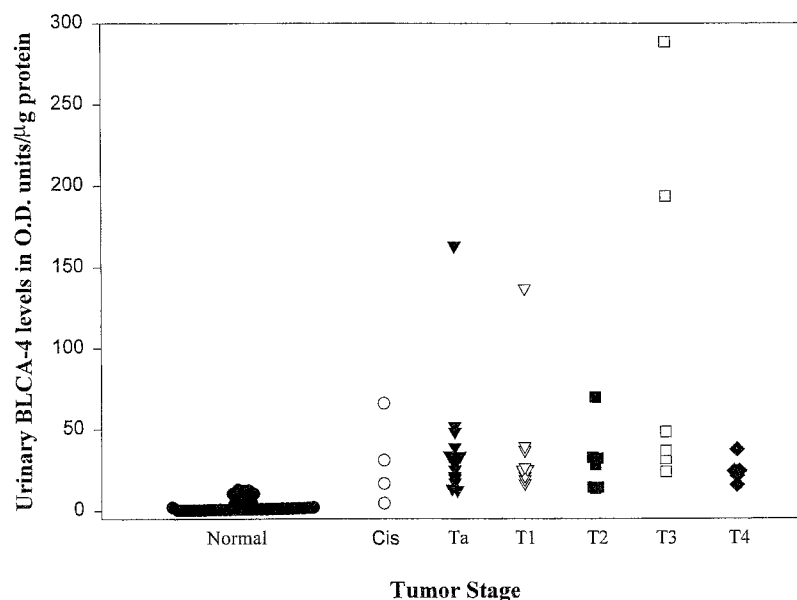


absorbance readings obtained with serial dilutions of urine samples containing added peptide also decreased in an approximately linear fashion.

The average value for BLCA-4 in the urine of the 51 normal control individuals was  $4.02 \pm 4.21$  A units/ $\mu\text{g}$  of protein, whereas the average value for individuals with bladder cancer was  $43.36 \pm 49.52$  A units/ $\mu\text{g}$  of protein. The levels are significantly higher in patients with bladder cancer ( $P = 2.2 \times 10^{-18}$ ). The cutoff value of 13 A units/ $\mu\text{g}$  of protein yielded a sensitivity of 53 of 55 (96.4%; CI, 87.5–99.6%) and a specificity of 51 of 51 (CI, 93–100%). There was no clear evidence of an association between BLCA-4 and

tumor grade ( $P = 0.11$ ), although the highest five urinary BLCA-4 levels occurred in patients with grade 3 tumors (Fig. 3). Elevated urinary BLCA-4 levels were present in early-stage tumors, including two of three carcinoma *in situ* and 11 of 12 with stage Ta (Fig. 4). Voided urine cytology reports were available in 32 of the 54 patients with bladder cancer. Of these, 16 patients had positive urine cytology, 5 were interpreted as suspicious, 4 were read as atypical, and 7 were negative. Only 2 of these 32 patients had a urinary BLCA-4 level below the cutoff of 13 A units/ $\mu\text{g}$  protein, and the urine cytology of one of these patients was interpreted as negative, whereas the other patient was positive. Hence, using urine

**Fig. 4** Distribution of urinary BLCA-4 levels according to stage of tumor resected. Four of the 54 patients whose urine samples were analyzed had carcinoma *in situ*, 16 had stage Ta tumors, 9 had T<sub>1</sub> tumors, 7 had T<sub>2</sub> tumors, and 6 each were of stage T<sub>3</sub> and T<sub>4</sub>, respectively. All samples from normal individuals had BLCA-4 levels <13 A units. The precise stage could not be determined in six individuals because of inadequate tissue samples.



cytology alone, 16 of 32 patients (50%) would have had an equivocal or negative diagnosis. Of these 16 patients, BLCA-4 levels were positive in 15 (93.7%). The BLCA-4 levels were also abnormally elevated in 15 of 16 of the individuals with positive urine cytology. BLCA-4 could therefore identify even those individuals in whom bladder cancer is not diagnosed by urine cytology. We anticipate that the accuracy of BLCA-4 in detecting all individuals with bladder cancer will improve with the development of monoclonal antibodies and further refinement of the detection assay technique.

For the urine-based BLCA-4 analysis, the volunteers were considerably younger than the patients (mean, 47 *versus* 71 years). To examine the possibility of confounding, the analysis was restricted to the overlap in the two age ranges (45–81 years). This resulted in age balance (mean, 62 *versus* 67 years). Using the cutoff of  $A = 13$  units/μg protein, the sensitivity was 97.7% ( $n = 43$ ; CI, 87.7%–100%), and the specificity was 100% ( $n = 22$ ; 95% CI, 84.6%–100%). There was no evidence of an effect of age on BLCA-4 ( $P = 0.17$ ).

## Discussion

This study demonstrates that antibodies can be raised against a peptide sequence from a bladder cancer-specific NMP (BLCA-4). The immunoblot studies demonstrate that the anti-BLCA-4 antibodies are able to distinguish the bladder tissue of individuals with bladder cancer from the bladder tissue of those without the disease. Furthermore, these data support the presence of a field effect in the bladder, suggesting that even cells that appear to be morphologically normal have undergone alterations. The fact that anti-BLCA-4 antibodies are able to identify “normal” tissues in every patient with bladder cancer suggests that these antibodies may be able to detect very early lesions of bladder cancer, even prior to morphological alterations.

One question that is raised by these studies is why the BLCA-4 protein, which was originally described as a NMP that distinguished tumor samples from normal samples of patients with bladder cancer by two-dimensional electrophoresis, is found in the normal bladder tissue of patients with bladder cancer by immunoblot. The data currently available do not address this discrepancy. One possibility is that the protein sequence that we have generated and the antibodies that we have raised are not to the original BLCA-4 protein. Our data argue against this possibility. This is supported by two-dimensional immunoblot analysis demonstrating the ability of the antibody to detect the spot (data not shown). Our current hypothesis is that the BLCA-4 protein was present in the two-dimensional gels of the normal surrounding tissue from patients with bladder cancer. This would not be surprising considering that bladder cancer is thought to be a field-change phenomenon. Very low levels of BLCA-4 expression could conceivably occur, even in morphologically normal areas of the bladder, in patients with bladder cancer. These gels are silver stained, and it is possible that something in these samples interfered with the silver staining process. The antibody we generated in this study is able to detect much lower quantities of the protein, which may not have been visible on the original silver stained two-dimensional gels. The detection of BLCA-4 in immunoblots of the “normal”-appearing mucosa from bladder cancer patients could also negatively affect interpretation of this test in patients with previously treated bladder tumors who are being monitored for recurrence. In such patients, the trend or absolute level of BLCA-4 in the urine may provide more information than predictions based solely on its presence or absence. This has proved to be the case with other markers such as serum prostate-specific antigen. We are in the process of analyzing urine samples from patients who have received prior therapy for bladder cancer to determine changes in BLCA-4 levels

before, during, and after surgery or intravesical immuno/chemotherapy for bladder cancer.

Elevated urinary levels of another generic NMP not specific to bladder cancer have been detected in patients with bladder cancer using the NMP22 test. Patients with bladder cancer have been found to have levels that are 25-fold greater than those of normal individuals (24). Soloway *et al.* (22) have determined NMP-22 levels in bladder cancer patients after surgical resection to detect tumor recurrence. Values >10 units/ml of urine were considered positive. The NMP-22 test had a sensitivity of 69.7% and a specificity of 78.5% in these patients for predicting recurrent tumor. Landman *et al.* (25) investigated the accuracy of NMP-22 in detecting bladder cancer, using a different cutoff value of 7 units/ml. The lower normal cutoff value improved the sensitivity to 81%, and the specificity was relatively unchanged at 77%. In a multicenter trial, urine analysis was performed on >1000 patients treated previously for bladder cancer who were being monitored for recurrence of their disease (22). The NMP-22 test was able to detect all of the cases subsequently identified as having invasive disease and ~70% of the cases with localized recurrence. However, NMP-22 is not specific for bladder cancer, and it appears useful only to detect recurrence of the disease. The presence of cystitis results in spuriously high levels of NMP-22, which are similar to those found in patients with bladder cancer (26). In patients without a prior diagnosis of bladder cancer, NMP-22 had a greater sensitivity (80.9% *versus* 40%) but a lower specificity (64.3% *versus* 100%) than voided urine cytology (27). However, these values for sensitivity and specificity are still significantly lower than the values obtained using the BLCA-4 assay, further emphasizing the bladder cancer-specific nature of BLCA-4.

It is important to standardize measurements of the urinary level of BLCA-4 by comparing it to that of other urinary constituents, such as creatinine or sodium. Variations in urinary protein levels caused by changes in hydration, renal disease, and others will also impact upon urinary BLCA-4 levels. In the current study, we addressed this issue by determining the BLCA-4 level relative to the total amount of protein in each urine sample. Equal amounts of sample were used for each assay (50  $\mu$ l), and the absorbance readings were standardized relative to the protein concentration of that particular sample.

In conclusion, BLCA-4 appears to be a promising bladder cancer-specific marker. The BLCA-4 protein was found in both cancerous and normal tissue in the bladders of all tested individuals with bladder cancer but not in bladder tissue from organ donors without the disease. In addition, BLCA-4 is found in morphologically normal areas of the bladder in individuals with bladder cancer. This protein has not been found in other tissue or cancer types. The fact that it is found in grossly "normal"-appearing areas of the bladder from individuals with bladder cancer suggests that it may be useful in detecting early disease. The data presented here suggest that BLCA-4 may be able to serve as a urine-based marker with which to screen for and identify individuals with bladder cancer and is significantly more accurate than urine cytology alone in identifying patients with bladder cancer.

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