Characterization of the DD23 Tumor-associated Antigen for Bladder Cancer Detection and Recurrence Monitoring

Rebecca B. Bonner, Monica Liebert, Robert E. Hurst, H. Barton Grossman, Barbara L. Bane, George P. Hemstreet III, and the Marker Network for Bladder Cancer

Abstract
Bladder cancer detection, monitoring, and prevention represent major problems that could be addressed with sensitive and specific biomarkers. The antigen recognized by the DD23 antibody, previously developed against a tumor-related antigen, was partially biochemically characterized, and its sensitivity and specificity in cancer detection and recurrence monitoring was evaluated. Quantitative fluorescence image analysis was used to quantify antigen content in exfoliated urothelial cells in a cross-section of patients with bladder cancers of all grades and stages and control populations. The antigen was found in tumor cells as well as normal-appearing urothelial cells, suggesting it represents a marker induced by the altered growth factor environment of a cancer-containing bladder. When used as a quantitative marker, the sensitivity for bladder cancer detection was 85\%, and the specificity was 95\%. No significant difference was seen between symptomatic and asymptomatic control populations, including patients with previous bladder cancers in the absence of a recurrence. In bladder cancer recurrence monitoring, results were consistently negative until just before detection of a recurrence. The biomarker reflects a "field effect" that occurs very late in tumorigenesis and seems to represent events common to most cancers involving the genitourinary tract. Western blotting showed the antibody recognized a dimeric protein. DD23 quantification in single cells may be particularly useful in targeting cystoscopic intervention for recurrence monitoring and, because of its high specificity, could be a tool for bladder cancer screening in high-risk groups.

Introduction
Bladder cancer will affect an estimated 50,500 Americans and 59,200 Europeans in 1995, and 11,200 and 27,380, respectively, will die of the disease (1, 2). Approximately half of high-grade lesions have metastasized by the time symptoms of hematuria and dysuria appear (3). Although 85\% of bladder cancers are low-stage papillary tumors at initial diagnosis in the United States, 15\% of these eventually progress to dangerous high-grade disease with greatly increased risk of patient mortality, while many will recur (4). The high rate of recurrence and progression probably reflects the biology of the disease, which often results in development of "field disease," in which malignancies develop from wider areas of altered uroepithelium (4–6). Biochemical abnormalities in morphologically normal areas of mucosa can be used to detect alterations in the field and are referred to as "field markers" (6). Field markers can be separated into "field disease markers," which are markers that are positive in premalignant cells, and "field effect markers," which reflect an effect of abnormal cells on cells that are genetically unaltered.

Because of insufficient sensitivity or specificity of current laboratory tests, monitoring for bladder cancer recurrence is primarily by invasive cystoscopy, which may fail to detect small high-grade flat carcinomas and carcinoma in situ (7) and is completely insensitive to upper-tract disease. Bladder cancer is concentrated in several high-risk groups including smokers and workers in certain occupations (8). In these high-risk groups, Papanicolaou cytology is ineffective in detecting cancer early enough to alter eventual unfavorable outcomes in this cohort (9). Clearly, alternatives for screening high-risk groups for bladder cancer and monitoring bladder cancer patients for recurrence are needed.

The previously developed DD23 antibody against human bladder cancer (10) was clinically evaluated for cancer detection and recurrence monitoring with exfoliated urothelial cells in a study design previously developed to efficiently identify markers with high predictive value (11, 12). The design incorporates several defined study populations, including cancer cases, asymptomatic controls, and symptomatic controls from a urological clinic to identify potential confounding conditions and provides estimates of sensitivity and specificity in populations similar to those in which a test would be used. To further support the use of the antigen as a marker, it was partially characterized biochemically, and the conditions under which bladder cancer cells express the marker in vitro were investigated.

Patients and Methods

Patient Population
Exfoliated urinary cell samples were collected from bladder cancer patients, symptomatic controls, and asymptomatic controls using the design published by Bonner et al. (11). Four main groups of patients and controls were analyzed: (a) asym-
tomatic controls; (b) symptomatic controls; (c) patients with bladder cancer; and (d) patients being monitored for recurrence of bladder cancer. Asymptomatic controls (n = 41) were frequency-matched by age and sex with bladder cancer patients and selected to be a healthy population with minimal possible confounding variables. The mean age was 58 years; 26 were current smokers or had smoked heavily in the past, and 15 were nonsmokers; 31 were male, and 10 were female. Each control subject was administered the Standardized Symptom Index for benign prostatic hyperplasia developed by the American Urological Association (Abbott Laboratories, publication #312-501-2889D, Abbott Park, IL: Ref. 13) to exclude patients with bladder outlet obstruction. Exclusion criteria were: (a) a symptom score of greater than 5; (b) symptoms of bladder outlet obstruction within 2 months of collection; (c) occupational exposure to known carcinogens; (d) age less than 46 years; (e) current cancer or a history of cancer other than superficial basal cell carcinoma; or (f) previous radiation or chemotherapy. The symptomatic control group included patients with a variety of urological conditions including chronic cystitis (5 patients), benign prostatic hyperplasia (15 patients), prostatic carcinoma, post-transurethral prostatectomy (5 patients), prostatitis (2 patients), urethral diverticulum, interstitial cystitis, and Peyronie’s plaque and impotency (1 patient each), and unknown diagnosis (4 patients) who were seen primarily at the urology clinics at Veterans Administration Hospital and The University Hospital in Oklahoma City, Oklahoma. Exclusion criteria were: (a) age less than 46 years; (b) lack of a definitive clinical diagnosis; (c) unexplained hematuria; (d) a history of cancer other than basal cell carcinoma; or (e) previous radiation or chemotherapy. Because the intent with these samples was to identify confounding medical conditions, the symptomatic controls were not matched for demographic variables. Bladder cancer cases included carcinoma in situ and TCC1 (7 grade 1, 11 grade 2, 13 grade 3, and 2 papillary Ta tumors), squamous cell carcinomas, and small cell carcinomas (Table 1). The group consisted of 32 males and 9 females, with an average age of 61 years. The cases were obtained mainly from the centers participating in the National Bladder Cancer Network at the University of Michigan and the University of Oklahoma, with some samples contributed by the University of Rochester, the University of Laval, and the University of California at San Francisco.

Table 1 Case mix and percentage of selected groups testing positive using mean DD23 content as the response variable

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>N</th>
<th>Percentage positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asymptomatic age-matched controls</td>
<td>41</td>
<td>4.9</td>
</tr>
<tr>
<td>Symptomatic controls</td>
<td>34</td>
<td>8.8</td>
</tr>
<tr>
<td>Bladder cancers</td>
<td>41</td>
<td>85.4</td>
</tr>
<tr>
<td>TCC</td>
<td>33</td>
<td>84.9</td>
</tr>
<tr>
<td>Carcinoma in situ</td>
<td>5</td>
<td>80</td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
<td>2</td>
<td>100</td>
</tr>
<tr>
<td>Small cell carcinoma</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Other cancers of the genitourinary tract</td>
<td>9</td>
<td>67.7</td>
</tr>
<tr>
<td>Prostate carcinoma</td>
<td>6</td>
<td>67</td>
</tr>
<tr>
<td>TCC of the renal pelvis</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Squamous cell carcinoma—metastatic</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Renal cell carcinoma</td>
<td>1</td>
<td>100</td>
</tr>
</tbody>
</table>

1 The abbreviations used are: TCC, transitional cell carcinoma; ROC, receiver operator curve.

Collection and Processing of Specimens

Urones or bladder washings were immediately fixed by adding electron microscope (EM)-grade formaldehyde (Polysciences, Warrington, PA) to achieve a concentration of 0.5% followed by an equal volume of 50% buffered ethanol after 15 min, as described previously (11). Samples from other institutions were shipped by overnight express. Urinary cells were concentrated by centrifugation and stored in 50% buffered ethanol at −85°C until processing in a bar code-labeled tube. After this point in the processing, laboratory personnel had no means of knowing the diagnosis of a particular patient.

Immunofluorescence Labeling

Slides were prepared for immunofluorescence as described previously (11). Briefly, the frozen cells were thawed and collected on an 8-μm polycarbonate 25-mm filter and fixed for 2 min with 2% polyethylene glycol 1450 in 50% buffered ethanol. Cells were transferred by imprinting the filter onto poly(l-lysine)- or silane-coated Probe-On microslides (Fisher Scientific, Houston, TX). The slides were then quickly sprayed with Carbofix-E (StatLabs, Inc., Lewisville, TX) and allowed to air-dry. Slides were stored at −20°C before analysis.

Slides were labeled for immunofluorescence using the computer-controlled Code-On immunostainer (Biotek Solutions, Santa Barbara, CA), which sequentially and reproducibly carries out a programmed sequence of labeling, incubation, and washing steps with automation buffer (Fisher Scientific). The DD23 antibody is obtained as a hybridoma cell-culture supernatant and is an IgG1 isotype (10). The slides were incubated for 30 min in the undiluted DD23 antibody-containing supernatant, washed 3 times followed by a 30-min incubation with biotinylated goat anti-mouse IgG (Life Technologies, Inc., Grand Island, NY) diluted in the automation buffer, and washed 3 times and incubated for 30 min with Neutralite (avidin)-conjugated Texas Red (Molecular Probes, Eugene, OR) diluted 1:500 and washed 5 times. The slides were finally incubated with aqueous 8.7 μM Hoechst 33258 (Polysciences) in 0.1 M NaCl, 0.05 M 2-hydroxy-3-morpholinopropanesulfonic acid (MOPSO) buffer, and 5 mM EDTA, with five changes of the solution to ensure saturation with a total incubation time of 6 min. Slides were mounted in 0.09 μM n-propyl gallate (Sigma Chemical Co., St. Louis, MO) in glycerol (Fisher Scientific). Control slides were incubated in each batch, consisting of a positive control for DD23 antibody (UM-UC13 cell line) and a negative control from each sample treated with 1:2000 mouse IgG (Pierce Chemical Co., Rockford, IL) instead of the primary DD23 antibody.

Development and Validation of Method

Several important methodological questions concerning the significance and validity of quantitative DD23 measurements require addressing to assess the significance of the clinical study. Optimal antibody concentrations are established by titration as described previously to ensure saturation of antigenic sites (14). The effect of fixation is investigated to ensure the epitope is not destroyed by fixation (15). Reproducibility is assessed as described below. A number of choices exist for selection of the response variable, and the choice is not intuitive. Examples include the mean fluorescence of some number of randomly selected cells, the fraction of cells above a threshold, or mean intensity of cells above a threshold (6). The choice of response variable is determined by analysis of the distribution function of cells. Because DD23 seems to be a field marker, two response
variables were tested: the mean DD23 content of 100 cells and the fraction of cells exceeding a threshold intensity. 

Quantitative Fluorescence Image Analysis. The slides were scanned with the Zeiss Axitron microscope (Thornwood, NY) at a final magnification of ×32 using a scanning stage and the IBAS image analysis system (Roche Image Analysis Systems, Elon College, NC). The IBAS automatically captures each field, segments each scene, and extracts morphometric features of individual cells (11). Fields were examined until approximately 100–200 nucleated epithelial cells had been measured by the instrument. The Texas Red emission was isolated by capturing an image of the emission using a quantitative Texas Red filter set (exciter 560 DF40, dichroic 595 DRLP, emission 630 DF23; Omega Optical, Brattleboro, VT) with a SIT camera (Hamamatsu, Bridgewater, NJ). The images were corrected for autofluorescence, and the mean gray level of each cell (G) was determined as the mean gray level of the pixels (p) on a scale of 0 (black saturation level) to 255 (white saturation level) comprising the cell image, with ni being the number of pixels.

\[ G = \frac{1}{n} \sum p 
\]

All the cell images were screened by an artifact rejection algorithm (11), and selected images with absolute DD23 values (see below) exceeding 85 units were further reviewed by a cytotechnologist.

The assay was standardized against an arbitrary standard of a cultured cell line (indicated with subscript "s" in the following equations) expressing the DD23 antigen (UM-UC-13 cells). The concentration of DD23 antigen/cell, in DD23 units (D), was calculated using Eq 2 and 3

\[ DD23 \text{ units/cell} = D_s = \frac{G_s}{G_i} \times 100 \]  

\[ G_i = \frac{1}{N_i} \sum G_{i,j} \]

where \( G_i \) is the population mean of all the standard cells, and \( N_i \) is the number of cells measured. The proportionality constant between immunofluorescence and biochemical content can be determined in principle by measuring the mean immunofluorescence of a population of cells and the mean content/cell by biochemical analysis.

Test reproducibility was assessed by thawing a second aliquot of cells remaining after the first analysis and repeating the assay on the thawed cells as described above for 10 selected samples. The 10 tumor samples analyzed in this way had been frozen at −80°C for up to a year before repeat analysis. The mix of samples was chosen to represent a variety of tumor types, including the four of the five false-negative cases.

Determination of Response Variable and Threshold for Positive/Negative. One response variable is simply the mean DD23 content of 100 cells. An alternative analysis consists of counting the fraction of positive cells present, as is common in immunocytochemistry. Examination of the histograms (Fig. 1) shows a continuum of distributions of the fluorescence signal, and some bladder cancer cases do not contain a discrete population of cells that could be clearly labeled as positive. With quantitative fluorescence, unlike immunocytochemistry, the definition of “positive” can be fixed in quantitative terms, although two thresholds must be determined to calculate sensitivity and specificity. The first threshold defines the intensity above which a cell is considered to be positive, and the second threshold is the fraction of positive cells in a sample required to classify a sample as test-positive.

To establish a quantitative threshold of a positive cell, cumulative frequency plots were generated at different cell-positive thresholds. Because of the importance of high specificity, the specificity of DD23 as a count marker was fixed at 94%, approximately the same as that achieved in the quantitative mode. The sensitivity associated with each cell-positive threshold was read from the respective cumulative frequency plots at the point at which specificity achieved 94% and was plotted against the cell-threshold, as shown in Fig. 3.

Optimal thresholds were determined by analysis of cumulative frequency and ROC plots (16). Cumulative frequency plots show the fraction of cases above a threshold value (sensitivity) and the fraction of controls below a threshold value (specificity) as a function of the threshold. Corresponding sensitivity and specificity values are plotted against each other to form the ROC plot, which displays the trade-off of sensitivity and specificity. The optimal threshold was generally represented by the threshold corresponding to the farthest excursion to the upper right hand corner of the ROC plot. An ideal ROC plot in which normal and abnormal populations showed no overlap would display a slope of zero at 100% specificity until the optimal threshold is reached, at which point the slope becomes infinite, corresponding to a vertical line at 100% sensitivity.

Biochemical Methods
The DD23 antigen was partially characterized to support the finding that it is a field effect marker expressed not only in cancer cells but in noncancer cells as well. The molecular weight of antigen purified by immunoprecipitation was measured by electrophoresis to determine whether the antigen was monomeric of dimeric. The antigen isolated from cells was probed with antibody under reducing and nonreducing conditions by Western blotting to determine whether the antibody recognizes a dimeric or monomeric form. Whether cell-matrix interactions enhance DD23 expression was determined by comparing levels of DD23 antigen in cells grown on plastic with those grown on Matrigel.

Cell Culture. Human bladder cancer cell lines UM-UC-9 and UM-UC-13 (10, 17) were maintained in MEM (Life Technologies, Inc.) supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, 8% newborn calf serum, and 2% FCS and cultured at 37°C in a humid incubator in a 5% CO2 atmosphere. Cells were grown on Matrigel (Collaborative Biomedical Products, Bedford, MA) using the thin gel method according to the manufacturer’s instructions. Cultured cells were recovered from the gel with Dispase and 10 mM EDTA as described by the manufacturer.

Western Blotting. Western blotting was performed using the method of Towbin et al. (18) with modifications as described previously (19). Briefly, UM-UC-9 cells were cultured in 6-well dishes, and after lysis with protease inhibitors (PBS containing 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM leupeptin, and 1% aprotinin) and centrifugation at 11,000 x g to remove nuclei, the extracts were mixed with either nonreducing or reducing sample buffer and separated on a 3% stacking and 7% separating polyacrylamide Laemmli gel (20) with prestained molecular weight markers (Rainbow: Amersham, Arlington Heights, IL). After electrophoretic transfer of the polyacrylamide gel to nitrocellulose, individual strips cut from the membrane were incubated overnight at 4°C with either DD23 or KJ23, a negative control antibody (Ref. 21; gener-
DD23 Bladder Cancer Marker

grown in 6-well plates and labeled overnight with culture as described previously (19). Briefly, UM-UC-9 cells were

OH) and developed with peroxidase-conjugated goat anti-

Fig. 1. Histogram of absolute DD23 units of cells exfoliated into urine from (front to back) an asymptomatic control, a patient with a TCC showing highly positive cells, and a patient with a TCC showing a shoulder of positive cells. UM-UC-13 cells were arbitrarily assigned a value of 100 units.

Immunoprecipitation. Immunoprecipitation was performed as described previously (19). Briefly, UM-UC-9 cells were grown in 6-well plates and labeled overnight with culture medium containing 160 μCi of [35S]methionine/ml (Amersham). After lysis and centrifugation, the extracts were incubated overnight at 4°C with either DD23 (test primary antibody) or KJ23 as the negative control primary antibody. Antibody-antigen complexes were separated under both reducing and nonreducing conditions on 7.5% microgels (Pharmacia, Piscataway, NJ) according to the manufacturer’s instructions. The gels were visualized with X-ray film (XAR; Kodak, Rochester, NY).

Results

Validation of Method. Fig. 1 presents histograms representative of an asymptomatic control and two bladder cancer cases representative of the kinds of distributions of DD23 in individual subject specimens. In the histogram from the asymptomatic control, few of the cells contained measurable DD23 antigen (about 80 units). In contrast, in the abnormal sample shown in the middle, virtually all the cells contained measurable antigen, with some cells containing extremely high levels of antigen. In the other histogram, the distribution appears to be comprised of two populations. Visual examination of labeled exfoliated cells from all samples disclosed both cancer cells and numerous normal-appearing cells labeled with the antibody, from which we concluded that the marker is expressed in the bladder field and not just in bladder cancer cells.

Long-term assay repeatability was assessed by remeasuring 10 selected samples. The mean of the values of the first 10 assays was 117 DD23 units and 108 DD23 units for the repeat 10 assays. A paired t test showed \( P = 0.595 \) (two-tailed), showing that the differences between the first and second tests are not statistically significant. The variances were 2642 and 2188, respectively, in the two sets of analyses. Two of the original false-negative results (see below) were positive on the second analysis, but no sample that was originally positive was negative on the repeat analysis. The original test results were used to calculate sensitivity and specificity.

Fig. 2 illustrates the establishment of a threshold for sensitivity of a positive cell. As described in "Patients and Methods", this calculation requires some assumptions based upon cumulative frequency and ROC-plot analysis (Figs. 3 and 4). When used as a count marker, the sensitivity drops off rapidly as the threshold for a positive cell is raised above 90–95 units. Shifting the threshold upward to label only bright cells as positive resulted in distinctly decreased sensitivity, and setting the threshold at 150 DD23 units, a level probably easily discernible by immunocytochemical techniques, resulted in a sensitivity of 67%.

The cumulative frequency and ROC curves are shown in Figs. 3 and 4, respectively. Visual examination of these plots suggests that thresholds of around 85 DD23 units or 35% of cells positive are optimal in separating abnormal and normal samples. This is confirmed in the ROC plot (which does not show the threshold), in which the optimal sensitivity and specificity correspond to a threshold of 85 DD23 units or 35% of cells positive. These test thresholds result in a sensitivity of 85% and specificity of 95% among asymptomatic controls (Fig. 4; Table 2) for both response variables. Also significant was the observation that the symptomatic and asymptomatic controls were essentially indistinguishable, and the initial slope of the control samples in the ROC plot was near zero.

Clinical Characteristics of DD23 Marker. Table 1 summarizes the percentage of positive test outcome (based upon quantitation) of the case mix analyzed. DD23 was positive in all grades of TCC, carcinoma in situ, and one small cell carcinoma case. Two false-positive test results were seen in the asymptomatic control group. Two false-positive test results were also identified in the symptomatic control group (both in men having bladder outlet obstruction, a known confounding variable of other bladder cancer markers; Ref. 11). No follow-up was performed on the asymptomatic controls, but a low American Urological Association Symptom Score was an inclusion criterion. Of great interest was the observation that the marker was positive in exfoliated cells from patients with other kinds of genitourinary cancers, including prostate, kidney, and squa-
mous cell carcinoma metastatic to the bladder. The low rate of false positives precluded a detailed analysis of confounding variables, although the low rate suggests smoking and other variables will have, at most, small effects. Examination of the five false-negative DD23 results showed they were near the median of the asymptomatic controls and not on the borderline. The false negatives included all grades of TCC: one each of grade 1 (bladder wash) and grade 3 (catheterized urine), two of grade 2 (both bladder washes), and one stage Ta of unknown grade (catheterized urine).

In bladder cancer patients being monitored for recurrence, the marker remained negative until shortly before recurrences were detected. Three patients were monitored with periodic sampling for up to a year, as shown in Table 2. In the two cases with bladder cancer recurrences, DD23 was not positive until just before (8 and 20 weeks) detection of the recurrence by cystoscopy. However, the immediately preceding samples were consistently negative, and no trend of increasing values was observed in the preceding samples. Table 3 shows results for patients being monitored for recurrence from whom only a single sample was analyzed. Of the 14 positive samples, all but 1 was associated with a tumor recurrence. Of the four negative samples, three have not recurred, and one was a false negative with a grade 1–2 tumor.

**Partial Biochemical Characterization of DD23 Antigen.** Western blotting of UM-UC-9 cell extracts with the DD23 antibody (as shown in Lane 1 of Fig. 5A) revealed that under nonreducing conditions, a single band of approximately Mr 185,000 was observed. However, when the extract was separated under reducing conditions, no proteins were recognized by DD23 (Lane 2), indicating the epitope is lost upon reduction. Immunoprecipitation of UM-UC-9 extracts by DD23 resulted in isolation of proteins that under nonreducing separation yielded a major band migrating at Mr 185,000, with a minor band observed at approximately Mr 93,000 (as shown in Fig. 5B, Lane 1). Under reducing conditions, the Mr 185,000 band was nearly lost, and most of the immunoprecipitated proteins moved at approximately Mr 93,000 (Lane 2).
Few bladder cancer cell lines express significant levels of the DD23 antigen when grown on plastic, but they do when grown on Matrigel. On plastic, ScaBER, 5637, and T24 lines expressed a mean of 70, 24, and 48 DD23 units/cell. On Matrigel, the corresponding values were 105, 110, and 116 DD23 units/cell, respectively. Visual examination of other cell lines (including J82, RT4, and TCCSUP) showed they also expressed increased DD23 antigen when grown on Matrigel.

Discussion

In this publication, use of the DD23 monoclonal antibody with quantitative fluorescence image analysis for the detection of bladder cancer in exfoliated cells is described. The optimal sensitivity and specificity of 85% and 95% were achieved in a mixture of low- and high-grade urothelial carcinomas and included several Ta/T1 tumors. The high specificity observed in these initial studies (Phase IIb), even among patients with a previous history of bladder cancer, is unusual and suggests the marker should be evaluated in prospective Phase III trials.

The antigen is expressed in cells that are not apparently cytologically aberrant and is also expressed in bladder cells in response to cancers at other sites (e.g., prostate, renal cell carcinoma, and a squamous cell carcinoma metastatic to the bladder). Additionally, the antigen is often expressed in large numbers of cells (Fig. 3b), well in excess of the few percent of tumor cells identified with mutant p53 markers as being found in urine (23). These findings are consistent with the marker representing a response of the bladder epithelium to signals from transformed cells rather than being a marker expressed by cells undergoing tumorigenesis. DD23, then, is a marker for a field effect rather than field disease per se. A marker for field disease would be positive only in malignant and premalignant cells, whereas a marker for a field effect would be positive in cells that have not undergone genetic initiation but are only responding to the altered cytokine environment in the bladder.

Most other markers show a higher false-positive rate with symptomatic patients, although their specificity with asymptomatic patients and their sensitivity to disease may approach that seen with DD23 (24-28). The high specificity is also seen in patients with previous bladder cancers, and in only one case has a positive DD23 result not been followed by a recurrence within a year. The combination of M344 tumor-related antigen and aberrant DNA ploidy as measured by the prevalence of cells with >5C DNA (11) shows a 20-25% higher rate of positives in the absence of tumor recurrence. These markers identify rare cells in the urine and may in fact reflect field disease, i.e., the presence of cells progressing toward tumorigenesis. In contrast, the DD23 marker does not detect rare cells, either as a quantitative marker or a count marker. The field disease markers become positive well in advance of tumor recurrence and are mainly useful in identification of risk, i.e., the group of patients likely to eventually develop recurrences. Patients found to be at risk might be monitored more frequently, particularly if the DD23 marker were positive. The finding of a positive DD23 heralds a sharply increased probability of finding a malignancy in the urinary tract, including the upper tract or prostate. Far from being a disadvantage, this sensitivity to cancer at other sites in the urinary tract will increase the usefulness of the test. Additional investigation of the sensitivity to other genitourinary cancers and other potentially confounding variables such as stone disease seems warranted.

The biochemical data support the hypothesis that DD23 is a marker of a field effect. Western blotting of UM-UC-9 human bladder cancer extracts indicated that the DD23 antibody identifies a Mr 185,000 antigen under nonreducing conditions, but the antigen is not bound by antibody after reduction. However, immunoprecipitation resulted in isolation of a Mr 185,000 protein and a minor component of Mr 93,000 under nonreducing conditions that shifted to Mr 93,000 with a very minor component of Mr 185,000 under reducing conditions. These results indicate the antigen is a dimer and that the Mr 93,000 monomer is not recognized by the antibody. The monomers are the same size, suggesting that the antigen is a homodimer. The reason for the high specificity may derive from the antibody only binding to the dimeric form, which must not be present in cells derived from the normal bladder environment.

Cancer cells in the bladder alter the underlying stroma, which in response alters the overlying epithelium (6, 29). The antigenic dimer in the cytoplasm may form in response to an external stimulus, possibly from paracrine signals from the stroma, as suggested by the enhanced DD23 levels seen in tumor cells grown on Matrigel. In UM-UC-13 cells, the response is probably autocrine because they express DD23 constitutively in the absence of stromal stimulation. Other factors are also involved in regulating the expression of DD23 antigen because even cells grown on Matrigel do not express the levels of DD23 antigen observed in some tumor cells. The finding that the dimer is also expressed in bladder cells in response to other cancers in the genitourinary system suggests an endocrine mechanism may operate as well. The dimeric marker is also expressed equally in low- and high-

<table>
<thead>
<tr>
<th>Table 3 Follow-up results on patients monitored for recurrence of TCC from whom only a single sample was analyzed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Follow-up results</td>
</tr>
<tr>
<td>Tumor concurrent with collection</td>
</tr>
<tr>
<td>Recurrence within 6-12 months</td>
</tr>
<tr>
<td>No recurrence</td>
</tr>
<tr>
<td>Lost to follow-up</td>
</tr>
<tr>
<td>Total</td>
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</table>
grade urothelial cancers including pure carcinoma in situ, although invasive and noninvasive papillary tumors seem to develop along different genotypic tracks (30, 31). The formation of the antigenic dimer is highly restricted because it is detected in very few normal tissues (22) and, as shown in this communication, is not strongly associated with conditions such as outlet obstruction that correlate with expression of other markers (11). One possibility is that DD23 represents a second messenger in one of the many cell-signaling mechanisms identified to date (32–37), with the dimeric form being induced by the binding of a ligand to an unknown cell-surface receptor.

The marker may well be useful for screening or monitoring high-risk groups. The lifetime risk for bladder cancer is about 3.2% for males and somewhat lower for females (38). The rate rises sharply with age, and the disease is much more prevalent in older populations. The low prevalence of bladder cancer demands a test with a high specificity and sensitivity to be cost-effective compared to that of other cancers such as prostate and breast. The incidence of bladder cancer in the general population is estimated at 3 per 10,000 (1) and increases to at least 13 per 10,000 in smoking males age 55 and older (39, 40). In high-risk cohorts, the incidence increases to as high as 40–50 per 10,000 (9, 12). Preliminary studies with 14-day dipstick screening found a prevalence of TCC of 130 per 10,000. (39) Dipstick screening is an inexpensive test that can be performed at home but suffers from a poor specificity with a false-positive rate of 16–20% for bladder cancer, with half of these having no detectable urological disease of any kind (39). Using a specificity of 95% and sensitivity of 85%, out of 10,000 screened, the total expected positives would be some 111 (130 x 85%) true positives, using Messing’s figures for the prevalence of cancer in such high-risk populations (39), and 500 false-positives, for a positive predictive value of 22%. This value is comparable to the positive predictive values of any other screening test, including prostate-specific antigen, mammography, or cervical cancer screening. The test can also be performed on a single voided urine that can be fixed and shipped to a central laboratory.

Although other biomarkers may have similar sensitivity as DD23 (41), the specificity of DD23 is much higher than other biomarkers, particularly in symptomatic patients and patients being monitored for bladder cancer recurrence. Symptomatic patients are the population in which biomarkers are most commonly applied. The DD23 marker fills an important gap in emerging biomarker profiles, particularly for recurrence screening. It occurs late in the process of tumorigenesis (thereby minimizing false positives from residual field disease), is highly specific for malignancy, and occurs in a high percentage of cases. If additional studies confirm the sensitivity and specificity found in this small-scale study, the marker could fill an important clinical need. It is important to emphasize that immunohistochemistry is not likely to yield the same high sensitivity (Fig. 3) as shown by quantitation of either mean DD23 content/cell or counting positive cells against a quantitative threshold.

In summary, this study identifies a quantitative marker with high sensitivity and specificity in the detection of bladder cancer. The marker seems to identify a field effect rather than field disease. It seems to detect malignancies in the genitourinary tract while being virtually insensitive to many known confounding conditions such as smoking, bladder outlet obstruction, and a previous history of bladder cancer. The appearance of the marker just before recurrence of a clinically detectable tumor suggests DD23 should also be useful for monitoring bladder cancer patients. The sensitivity and specificity suggest quantitative DD23 measurements as a method potentially applicable to screening asymptomatic but high-risk populations after further investigation to identify additional confounding variables and establish sensitivity and specificity in the targeted populations.

References


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