Evaluation of Two New Urinary Tumor Markers: Bladder Tumor Fibronectin and Cytokeratin 18 for the Diagnosis of Bladder Cancer

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ABSTRACT
Our objectives were to evaluate the diagnostic value of two new urinary tumor markers, cytokeratin 18 (CK18) and bladder tumor fibronectin (BTF), for the detection and monitoring of bladder cancer. The study comprised 931 urine samples belonging to 402 subjects: 112 individuals under suspicion for a primary bladder tumor (group 1); 104 bladder cancer patients under scheduled follow-up (group 2); 109 bladder cancer patients receiving intravesical instillations (group 3); 45 patients with other urological diseases (group 4); and 32 healthy subjects (group 5). Voided urine samples were collected before cystoscopies, between them and before intravesical instillations. CK18 and BTF tests were measured by chemiluminescent immunoassays. Optimal receiver operating characteristic cutoffs of 7.4 μg/L for CK18 and 52.8 μg/liter for BTF rendered overall sensitivities of 66.2% for CK18 and 80.0% for BTF at specificities of 88.4% and 74.7%, respectively. Urinary cytology provided a sensitivity of 29.2% at a specificity of 99.1%. Sensitivities were 80.8, 74.2, and 82.3% for BTF and 71.1, 77.4, and 64.7% for CK18 for groups 1 to 3, respectively. False positive rates were higher for BTF in all groups of patients. Elevated urinary tumor markers during the monitoring of patients with bladder cancer could detect recurrence sooner than scheduled cystoscopies. Persistence of negative markers was greatly indicative of free of disease status in follow-up. CK18 and BTF in urine may eventually prove to be of benefit for specific patients with bladder carcinoma given its higher sensitivity compared with cytology. In selected patients, namely those with persistent negative urinary CK18 and BTF, the number of cystoscopies could be reduced.

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INTRODUCTION
The low sensitivity of cytology necessitates frequent cystoscopy under scheduled follow-up protocols for the surveillance of patients with bladder cancer (1–3). As a result, the search for alternative objective noninvasive methods for the detection of bladder cancer is warranted. Different urinary tumor markers for the diagnosis of bladder cancer are being explored in the last few years (4–9), even more since the approval of BTA3 (4), NMP22 (5), and fibrinogen degradation products (7) by the Food and Drug Administration for the detection of recurrence in patients with bladder cancer (3). In this study, our main objective was to evaluate the potential role in the diagnosis and monitoring of the disease of two recently developed urinary tumor markers, a new test to quantify CK18 and a new tumor marker for the measurement of fibronectin in the urine, namely BTF. We reported one year-long follow-up monitoring of patients under risk for bladder cancer with serial urinary tumor markers aiming to define the situations in which their measurement would be most beneficial and to assess whether the information provided was efficient enough to be introduced into daily clinical practice and individualize intervals between cystoscopies.

CKs are differentiation intermediate filament proteins which constitute the cytoskeleton of epithelial cells (10, 11). They have been found in bladder urothelium, and their potential value has been suggested as tumor markers for bladder malignancy (12–14). Different CK markers have been evaluated for bladder cancer diagnosis in urine samples, such as urinary bladder antigen (measuring urinary fragments of CK8 and CK18) (8, 15), CYFRA 21-1 (quantifying CK19) (16–18), or tissue polypeptide-specific antigen (determining CK18) (19). A new chemiluminescent enzyme-labeled immunometric assay with a monoclonal antibody binding to a region close to the CK18 M3 epitope (20) has recently been launched. We were interested in exploring its diagnostic value aware of the already described ability of different CKs alone or combined with CK18 for the detection of bladder cancer (8, 15–18).

Fibronectin is a 440-kDa large dimeric glycoprotein that is present in the extracellular matrix. Through its series of functional domains, it can bind to the cell surface and recognize various biological molecules such as fibrin, collagen, DNA,
heparin, and other connective components (21). Fibronectin is believed to be involved in the interaction of cells with the extracellular matrix (22): cell-to-substrate adhesion, cell migration, and regulation of cell morphology (21–24). It has been found in plasma (25–27), biological fluids such as the urine (28–30), and tissues (31, 32), including the urinary tract epithelium (31, 33, 34). The presence of fibronectin and fragments in urine appears to be related to proteolytic degradation by enzymes produced by tumor bladder cancer cells (35–39). Actually, urinary fibronectin has already been found to be elevated in patients harboring a bladder tumor as compared with patients free of disease (38–40). Different polyclonal and monoclonal antibodies against fibronectin N-terminal, COOH-terminal, and cell-binding domains have already been described (31, 37–41). Although lack of specificity of fibronectin as a tumor marker in plasma and tissue has been reported for different malignancies (26, 27, 31, 32), we were interested in evaluating the diagnostic ability of this new test using an antibody against the cell-binding domain of the molecule to quantify fibronectin fragments in the urine for the detection of bladder cancer.

MATERIALS AND METHODS

Patients, Samples, and Study Design. The study encompassed 402 subjects. Patients under suspicion for bladder cancer who were to undergo cystoscopy over the period of study were classified into 3 groups: group 1 comprised 112 subjects with microhematuria under suspicion for a primary bladder tumor; group 2 included 104 follow-up patients with a previous bladder cancer under scheduled follow-up controls; group 3 consisted of 109 bladder cancer patients receiving intravesical instillations of chemotherapy with mitomycin C (n = 76) or Oncothiotepa (n = 14), or immunotherapy with BCG (n = 19). We also included an additional reduced control group consisting of 45 patients with benign and other urological diseases (group 4) and 32 healthy subjects (group 5).

During 1 year, 931 voided or catheterized urine samples were collected, centrifuged at 1500 rpm for 7 min at 4°C, aliquoted, and frozen at −20°C until each tumor marker measurement. Voided urine samples were collected before cystoscopy for patients of groups 1–3. Follow-up patients of groups 2 and 3 were monitored through urinary tumor markers. Not only did those of group 2 provide urine samples before the scheduled cystoscopies every 3 or 6 months but at least once or two additional monthly samples were collected between cystoscopies. Additionally, urine voided samples were collected weekly and/or monthly immediately before the chemotherapy instillations for patients of group 3. Only patients of groups 2 and 3 who provided a minimum of six urine voided samples and who underwent at least a final cystoscopy over the year of study were considered for the follow-up evaluation of urinary tumor markers in the monitoring of the disease. In a reduced number of patients, urine samples were collected when possible on the previous day or immediately before the surgical treatment to those patients whose cystoscopies had already revealed the presence of a bladder tumor. Patients and subjects from groups 4 and 5 were consecutively recruited during 3 months. All patients participating in the study were informed and gave their consent following the procedures approved by the ethical committee of our institution.

Demographic and Clinical Data. Demographic data such as age and sex were registered. The mean age in years ± SD (range) of subjects of each group was 66.0 ± 10.4 (35.0–89.0), 67.1 ± 8.8 (46.0–93.0), 68.2 ± 9.2 (38.0–87.0), 70.2 ± 9.8 (23.0–87.0), and 63.0 ± 11.4 (23.0–80.0), respectively, for groups 1–5, which were not statistically different (Kruskal-Wallis H = 8.49, P = 0.061). The female: male proportions were 25:87, 16:88, 15:94, 20:24, and 14:18, respectively. Urinary CREA mean concentrations (in mg/dl) ± SD (range) among groups were compared. These were 108.4 ± 59.5 (11.8–348.2), 110.1 ± 58.9 (10.7–257.5), 120.8 ± 62.3 (11.8–349.4), 130.1 ± 62.9 (15.3–339.4) and 128.1 ± 45.9 (55.9–246.9) in groups 1 to 5, respectively, and were not significantly different (Kruskal-Wallis H = 5.97, P = 0.114).

Patients with positive cystoscopies received surgical treatment by transurethral resection or cystectomy. Histopathological data were recorded. Cases of groups 1, 2, and 3 were staged as superficial (pTa, pT1) or muscle invasive (pT2, pT3, pT4) according to the tumor-node-metastasis criteria (42). No patient with incident or recurrent carcinoma in situ was detected in our series during our study from whom we could collect diagnostic urine samples to be included into the study. Grade was assessed following the World Health Organization tumor grading system (43). Patients who received chemo- or immunotherapy followed one of these alternative protocols: four weekly instillations followed by six or nine monthly instillations of mitomycin C, Oncothiotepa of BCG, or six weekly instillations for BCG. Urinary cytology and cystoscopy were performed every 3 months over the first follow-up year or every 6 months during the second year follow-up in patients from groups 2 and 3. Despite cytology, urine sample collection was performed independently from our study, and cytological findings scheduled simultaneously to cystoscopy were registered for comparison with urinary tumor markers.

Laboratory Methods. BTF and CK18 were determined by solid phase, two-site chemiluminescent immunometric commercial diagnostic assays in an IMMULITE Automated Immunoassay System provided by Diagnostic Products Corporation (Los Angeles, CA). The principle of the procedure is similar for both tests. The solid phase is polystyrene beads coated with murine monoclonal antibodies specific for CK18 or BTF, respectively, enclosed within a test unit specific for the analyzer. Whereas the urine sample and alkaline phosphatase-conjugated rabbit (for BTF) or donkey (for CK18) polyclonal specific antibodies are incubated for 30 min at 37°C with intermittent agitation, CK18 and BTF in the sample are bound to form an antibody sandwich complex. Unbound conjugate is then removed by a centrifugal wash, after which substrate is added and the test unit is incubated for further 10 min. The chemiluminescent substrate, a phosphate ester of adamantyl dioxetane, undergoes hydrolysis in the presence of alkaline phosphatase to yield an unstable intermediate. The continuous production of this intermediate results in the sustained emission of light. The bound complex and also the photon output as measured by the temperature-controlled luminometer are proportional to the level of CK18 or BTF in the sample. Test results are calculated.
from the observed signal, using calibration curves performed for each assay.

Two additional determinations were performed on all samples included into the study: (a) urinary CREA, which was measured by an enzymatic colorimetric method in the analyzer Synchron CX9 (Beckman, Los Angeles, CA); (b) all urine samples were submitted for urinalysis. Urinary blood, leukocytes, and nitrates were semiquantitatively determined by colorimetric methods with the use of Combur-Test M strips (Roche Diagnostics, Mannheim, Germany), which were read in a Miditron photometer (Roche Diagnostics).

**Analytical Evaluation of BTF and CK18 Tests.** Lower detection limits were defined through 10 consecutive measurements of standards of zero concentration. The precision of the Immulite BTF and CK18 tests was evaluated according to the National Committee for Clinical Laboratory Standards protocol EP5-T2 (44). Two point ligand controls and a pool from different human urine samples at clinically important analyte concentrations were analyzed 10 times a day for the intraassay variability study and daily for 10 different nonconsecutive days for 1 month for the interassay variability study. The linearity was evaluated through dilution of urine samples with different concentrations of BTF and CK18 with specific protein matrix urine diluents at 1/2, 1/5, and 1/10 dilutions of urine. The evaluations were made by the percentage differences between the expected and the observed values. To evaluate recovery, different amounts of a urine sample containing a high concentration of BTF and CK18 were added to urine samples at different concentrations. The evaluations were made by the differences between expected and observed values (recovery percentages).

**Statistical Analysis.** Optimal volumetric (micrograms/liter) and corrected by urinary CREA (micrograms/g) cutoffs were established by ROC curves analysis from 130 and 225 urines collected immediately before the performance of positive and negative cystoscopies, respectively. Urinary CREA cutoff to define the presence of a low concentrated urine was establish by the fifth low percentile in healthy subjects. An assumption of normal distribution was not possible in all of the groups of subjects; therefore, statistical inferences were evaluated by non-parametric tests. Differences between two means were performed according to the Mann-Whitney U test. When three or more groups were considered, differences were evaluated using Kruskal-Wallis nonparametric one-way ANOVA.

**RESULTS**

**Analytical Evaluation of BTF and CK18.** Lower detection limits were 0.2 and 0.03 μg/liter for BTF and CK18, respectively. The within-run coefficients of variation were 4.6, 4.2, and 3.2% at concentrations of 14.2 (control 1), 46.8 (urine samples pool), and 67.4 μg/liter (control 2) for BTF; and 4.6, 4.2, and 3.9% at concentrations of 2.2 (control 1), 6.9 (urine samples pool), and 12.4 μg/liter (control 2) for CK18. The between-day coefficients of variation were 4.9, 4.5, and 3.9% at concentrations of 14.5 (control 1), 46.2 (urine samples pool), and 67.9 μg/liter (control 2) for BTF; and 4.9, 4.8, and 4.2% at concentrations of 2.2 (control 1), 6.5 (urine samples pool) and 12.9 μg/liter (control 2) for CK18. Measurement of diluted urine samples showed the assays to estimate between 93 and 113% for BTF and between 92 and 103% for CK18. Recovery percentages ranges were 96–105% of added BTF and 96–115% for CK18.

**Diagnostic Profiles of Urinary Tumor Markers.** ROC analysis gave the optimal cutoffs of 7.4 μg/liter for CK18 and...
Table 1  Diagnostic characteristics of urinary tumor markers compared to urinary cytology, the presence of micro- and macrohematuria taking volumetric and normalized cutoffs given by ROC analysis in this series

Sensitivity was established from 130 urine samples belonging to patients with known bladder cancer collected immediately before cystoscopy and/or surgery. Specificity was taken from 225 urine samples belonging to disease-free patients collected immediately before cystoscopy.

<table>
<thead>
<tr>
<th>Markers</th>
<th>Cutoff</th>
<th>S (%)</th>
<th>Sp (%)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
<th>Acc (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BTF/CREA</td>
<td>52.8 µg/liter</td>
<td>80.0</td>
<td>74.7</td>
<td>64.6</td>
<td>86.6</td>
<td>76.6</td>
</tr>
<tr>
<td>BTF</td>
<td>77 µg/g</td>
<td>70.0</td>
<td>84.9</td>
<td>72.8</td>
<td>83.0</td>
<td>79.4</td>
</tr>
<tr>
<td>CK18</td>
<td>7.4 µg/liter</td>
<td>66.2</td>
<td>88.4</td>
<td>76.8</td>
<td>81.9</td>
<td>80.3</td>
</tr>
<tr>
<td>CK18/CREA</td>
<td>7 µg/g</td>
<td>64.6</td>
<td>88.0</td>
<td>75.7</td>
<td>81.1</td>
<td>79.4</td>
</tr>
<tr>
<td>BTF + CK18</td>
<td>52.8 µg/liter + 7.4 µg/liter</td>
<td>84.6</td>
<td>70.7</td>
<td>62.5</td>
<td>88.8</td>
<td>75.8</td>
</tr>
<tr>
<td>BTF/CREA + CK18/CREA</td>
<td>77 µg/g + 7 µg/g</td>
<td>85.4</td>
<td>76.9</td>
<td>68.1</td>
<td>90.1</td>
<td>80.0</td>
</tr>
<tr>
<td>Cytology</td>
<td>(+)/(-)</td>
<td>29.2</td>
<td>99.1</td>
<td>95.0</td>
<td>70.8</td>
<td>73.5</td>
</tr>
<tr>
<td>Microhematuria</td>
<td>50 µ/l</td>
<td>60.8</td>
<td>79.1</td>
<td>62.7</td>
<td>77.7</td>
<td>72.4</td>
</tr>
<tr>
<td>Macrohematuria</td>
<td>(+)/(-)</td>
<td>9.2</td>
<td>87.1</td>
<td>29.3</td>
<td>62.4</td>
<td>58.6</td>
</tr>
</tbody>
</table>

*S, sensitivity; Sp, specificity; PPV, positive predictive value; NPV, negative predictive value; Acc, global accuracy.

Table 2  Descriptive differential analysis of BTF and CK18 concentrations among all groups of patients with and without bladder cancer and subjects included in the study

<table>
<thead>
<tr>
<th>Groups</th>
<th>BTF Mean ± SD (95% CI)</th>
<th>SEM</th>
<th>Median (95% CI)</th>
<th>95% Percentile</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients with bladder cancer overall (N = 130)</td>
<td>293.0 ± 409.9 (221.9–364.2)</td>
<td>35.9</td>
<td>177.5 (124.0–276.0)</td>
<td>820.0</td>
<td>11.6–205.0</td>
</tr>
<tr>
<td>Patients with bladder cancer (group 1; N = 52)</td>
<td>288.9 ± 389.1 (180.6–397.3)</td>
<td>54.0</td>
<td>138.0 (112.9–400.0)</td>
<td>778.0</td>
<td>11.8–2009.0</td>
</tr>
<tr>
<td>Patients with bladder cancer (group 2; N = 31)</td>
<td>340.0 ± 469.9 (167.7–512.4)</td>
<td>84.4</td>
<td>271.0 (84.6–401.0)</td>
<td>1920.0</td>
<td>11.6–2034.8</td>
</tr>
<tr>
<td>Patients with bladder cancer (group 3; N = 17)</td>
<td>182.6 ± 151.8 (104.6–260.6)</td>
<td>36.8</td>
<td>125.0 (57.6–381.6)</td>
<td>407.0</td>
<td>22.0–2880.0</td>
</tr>
<tr>
<td>Patients without cancer overall (N = 225)</td>
<td>73.5 ± 200.3 (47.1–99.8)</td>
<td>13.3</td>
<td>29.0 (24.9–32.3)</td>
<td>304.7</td>
<td>0.4–2200.0</td>
</tr>
<tr>
<td>Group 4 (N = 45)</td>
<td>133.5 ± 142.0 (90.8–176.2)</td>
<td>21.2</td>
<td>51.0 (37.8–158.9)</td>
<td>401.0</td>
<td>9.1–410.0</td>
</tr>
<tr>
<td>Group 5 (N = 32)</td>
<td>32.5 ± 8.9 (28.4–36.6)</td>
<td>7.9</td>
<td>38.2 (29.2–48.5)</td>
<td>47.6</td>
<td>6.2–51.9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Groups</th>
<th>CK18 Mean ± SD (95% CI)</th>
<th>SEM</th>
<th>Median (95% CI)</th>
<th>95% Percentile</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients with bladder cancer overall (N = 130)</td>
<td>29.5 ± 43.2 (22.0–37.0)</td>
<td>3.8</td>
<td>13.8 (10.7–28.3)</td>
<td>59.0</td>
<td>0.1–259.0</td>
</tr>
<tr>
<td>Patients with bladder cancer (group 1; N = 52)</td>
<td>28.4 ± 37.8 (17.8–38.9)</td>
<td>5.2</td>
<td>15.4 (8.9–36.9)</td>
<td>52.0</td>
<td>0.5–240.0</td>
</tr>
<tr>
<td>Patients with bladder cancer (group 2; N = 31)</td>
<td>34.2 ± 44.9 (17.7–50.6)</td>
<td>8.1</td>
<td>28.6 (10.6–50.0)</td>
<td>66.1</td>
<td>0.8–251.0</td>
</tr>
<tr>
<td>Patients with bladder cancer (group 3; N = 17)</td>
<td>22.0 ± 20.8 (11.3–32.7)</td>
<td>5.1</td>
<td>12.4 (3.1–50.0)</td>
<td>73.0</td>
<td>0.1–276.0</td>
</tr>
<tr>
<td>Patients without cancer overall (N = 225)</td>
<td>5.0 ± 12.8 (3.3–6.7)</td>
<td>0.8</td>
<td>1.5 (1.3–2.0)</td>
<td>19.0</td>
<td>0.1–148.5</td>
</tr>
<tr>
<td>Group 4 (N = 45)</td>
<td>6.9 ± 9.7 (5.7–8.5)</td>
<td>1.4</td>
<td>3.4 (1.6–5.1)</td>
<td>24.9</td>
<td>0.1–56.0</td>
</tr>
<tr>
<td>Group 5 (N = 32)</td>
<td>3.6 ± 3.4 (2.4–4.3)</td>
<td>1.0</td>
<td>1.2 (0.9–1.5)</td>
<td>6.2</td>
<td>0.1–7.5</td>
</tr>
</tbody>
</table>

*a Numbers in parentheses, 95% CI.

52.8 µg/liter for BTF. These cutoffs rendered overall sensitivities of 66.2% (95% CI 57.8–74.7%) for CK18 and 80.0% (95% CI 72.1–86.5%) for BTF at specificities of 88.4% (95% CI 83.5–92.3%) and 74.7% (95% CI 68.8–80.5%), respectively. No statistical difference was found between normalized and volumetric ROC curves for BTF and CK18 as can be observed in Fig. 1. When ROC curves were compared among BTF and CK18, no statistical difference was found both in volumetric (P = 0.702) and in normalized units (P = 0.674). The diagnostic characteristics of BTF and CK18 with and without correction by urinary CREA are shown in Table 1. Urinary tumor marker diagnostic profiles were superior in all cases to the presence of micro- and macrohematuria and urinary cytology, this latter providing a sensitivity of 29.2% at a specificity of 99.1%. The combined determination of BTF and CK18 increased sensitivity to 84.6%, decreasing specificity to 70.7%, with positive and negative predictive values of 62.5 and 88.8%, respectively, and a global accuracy of 75.8%.

Defining 95% sensitivity as an optimal one, specificities of 26.2% (95% CI 20.6–32.5%) for CK18 and 24.4% (95% CI: 19.0–30.6%) for BTF, respectively, could have been obtained at the cutoffs of 0.9 µg/L for CK18 and 17.7 µg/L for BTF. At normalized cutoffs of 14.6 µg/g for BTF/CREA the specificity was 14.7% (95% CI: 10.4–20.1%) and at 1 µg/g for CK18/CREA it was 39.7% (95% CI 33.3–46.5%). Similarly, when we established 95% specificity as an optimal one, sensitivities of 26.2% (95% CI 20.6–32.5%) for CK18 and 14.7% (95% CI 10.3–20.0%) for BTF could have been obtained at the cutoffs of 18.5 µg/liter for CK18 and 247 µg/liter for BTF; when corrected by urinary CREA at cutoffs of 20.2 µg/g for CK18/CREA and 250.0 µg/g for BTF/CREA, the sensitivities would have been 48.5 (95% CI 39.6–57.4%) and 40.8% (95% CI 32.2–49.7%), respectively.

There were five patients whose cystoscopic findings suggested the presence of a bladder tumor. They were confirmed to be free of disease in their pathological examination of surgical biopsies. In these cases, urinary tumor markers were negative for bladder cancer in 2 of 5 for BTF, 4 of 5 for BTF/CREA, 4 of 5 for CK18 and 4 of 5 for CK18/CREA. The urinary cytologies of all these patients were negative as well.

Comparing precystoscopy urines versus preoperative urine samples in 17 patients from whom it was possible to collect...
urine samples in both situations, urinary tumor markers showed mainly the same negative or positive profile in both urine samples with different frequencies for each tumor marker. However, in 2, 2, 3, and 6 cases precystoscopy, urinary BTF, BTF/CREA, CK18, and CK18/CREA concentrations were below the cutoffs and increased above thresholds in urine samples collected immediately before surgery, the mean waiting time between cystoscopy and surgery being 26.3 days (range, 5–72 days). Positive urinary tumor markers before cystoscopy decreased to negative in preoperative samples in 2 and 1 cases for BTF and BTF/CREA, respectively.

Table 2 shows the descriptive analysis concentrations of BTF and CK18 in volumetric units taking together all patients with bladder cancer and separating each group of patients harboring a bladder tumor (group 1, group 2, and group 3) and considering together all those without bladder cancer whose clinical status was confirmed by subsequent cystoscopies. Urinary BTF and CK18 from control groups of other urological diseases and healthy subjects were included as well.

We analyzed the differential diagnostic characteristics of urinary tumor markers in each group of patients of groups 1 to 3 included in the study as referred in Table 3. Only urinary tumor marker results measured from urine samples collected immediately before cystoscopies for patients of groups 1, 2, and 3 were considered. Taking the different groups of subjects individually, BTF sensitivity was superior to CK18 in group 1. Of 52 incidental or screening cancers, urinary tumor markers could have detected the presence of the tumor in 42 of the cases for BTF (micrograms/liter) and 37 of the cases for CK18 (micrograms/liter). In follow-up patients without chemotherapy of group 2, sensitivities for both tumor markers were ~75%, slightly higher for CK18. Of 17 recurrences in patients receiving chemo- or immunotherapy, remarkably BTF could detect 14 of them and CK18 detected 11 cases of those that recurred. Normalization did not allow increase of the sensitivity of BTF and CK18 in any of the groups. However, the correction by urinary CREA raised their specificity, specially in group 3.

Significant statistical associations were found for both tests with stage and grade in both volumetric and normalized units. A descriptive analysis of concentrations and sensitivities of urinary tumor markers regarding stage and grade in volumetric and normalized units is shown in Table 4. We focused on identifying which tumors were detected with greater difficulty by urinary CREA. False negatives were 26 and 44 for BTF and CK18, all of which were superficial transitional bladder tumors except 4 patients for CK18 who harbored invasive disease. Normalization did not allow in this series to increase the sensitivities of urinary tumor markers in volumetric units.

Monitoring of the Disease with Urinary Tumor Markers. True elevated results of both urinary tumor markers simultaneously during the monitoring of patients with bladder cancer could detect recurrence sooner than scheduled cystoscopies in 14 patients of the 31 monitored patients of group 2 who developed a bladder cancer during the period of the study, as confirmed by cystoscopy. Additionally, early detection was shown individually in 13 cases for BTF (micrograms/liter) and for CK18 (micrograms/liter) and 13 and 14 for BTF/CREA (micrograms/g) and CK18/CREA (micrograms/g), respectively. Of 17 cases of monitored group 3 patients who developed
observed false positive results in 65; 58 cases for BTF and mitomycin C, Oncothiotepa and BCG, respectively) and 47 for CK18/CREA (3 of group 2, and 32, 4, and 8 receiving mitomycin C, Oncothiotepa, and BCG, respectively).

During monitoring of patients of groups 2 and 3, we observed negative results in both markers simultaneously in 50.9% of cases. Of the remaining 49.1%, 47.6% of patients showed persistence of negative results in both markers simultaneously, whereas 2.5% of them showed persistence of positive results in BTF and negative results in CK18. The most frequent factor associated with elevated results for CK18 was the presence of urinary tract infections. Additionally, the chemical cystitis associated with the initial weekly instillations in our current intravesical chemotherapy protocols was relevant for BTF/CREA; and 28 and 21 for CK18 and CK18/CREA. Overall false positive rates were significantly higher for BTF than for CK18 (P = 0.0005). Elevated nonexpected results were higher in patients of group 3 than in group 2 patients for BTF (59 versus 6), BTF/CREA (58 versus 5), CK18 (24 versus 4), and CK18/CREA (17 versus 4). Elevated results during the monitoring of the disease were higher for urine samples belonging to patients receiving intravesical chemotherapy or immunotherapy than for patients of group 2 and for BTF than for CK18. The most frequent factor associated with elevated results for CK18 was the presence of urinary tract infections. Additionally, the chemical cystitis associated with the initial weekly instillations in our current intravesical chemotherapy protocols was relevant for BTF in 25 patients in volumetric and normalized units, being the most frequently factor associated with high percentages of elevated results for BTF. Patients receiving BCG [10 of 19 (52.6%)] showed comparatively higher false rates associated to first instillations than those with mitomycin C [14 of 76 (18.4%)] and Oncothiotepa [1 of 14 (7.1%)]. Finally, one should not

<table>
<thead>
<tr>
<th>Stage</th>
<th>BTF (P = 0.0191)</th>
<th>BTF/CREA (P = 0.0018)</th>
<th>CK18 (P = 0.0001)</th>
<th>CK18/CREA (P = 0.0005)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTA (15)</td>
<td>136.7 ± 131.0</td>
<td>146.6 ± 156.6</td>
<td>69.7 ± 16.7</td>
<td>13.5 ± 28.1</td>
</tr>
<tr>
<td>10/15 (66.7)</td>
<td>8/15 (53.3)</td>
<td>4/15 (26.7)</td>
<td>4/15 (26.7)</td>
<td></td>
</tr>
<tr>
<td>PT1 (47)</td>
<td>250.0 ± 403.5</td>
<td>339.6 ± 629.4</td>
<td>22.5 ± 20.9</td>
<td>29.0 ± 34.8</td>
</tr>
<tr>
<td>34/47 (72.3)</td>
<td>29/47 (61.7)</td>
<td>35/47 (74.5)</td>
<td>34/47 (72.3)</td>
<td></td>
</tr>
<tr>
<td>PT2 (15)</td>
<td>646.6 ± 708.7</td>
<td>742.4 ± 872.1</td>
<td>77.2 ± 85.5</td>
<td>89.9 ± 106.6</td>
</tr>
<tr>
<td>15/15 (100.0)</td>
<td>14/15 (93.7)</td>
<td>13715 (86.7)</td>
<td>13/15 (86.7)</td>
<td></td>
</tr>
<tr>
<td>PT3 (3)</td>
<td>120.6 ± 51.7</td>
<td>114.9 ± 25.0</td>
<td>3.3 ± 3.9</td>
<td>3.0 ± 2.6</td>
</tr>
<tr>
<td>3/3 (100.0)</td>
<td>3/3 (100.0)</td>
<td>1/3 (33.3)</td>
<td>0/3 (0.0)</td>
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</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Grade</th>
<th>BTF (P = 0.0119)</th>
<th>BTF/CREA (P = 0.0352)</th>
<th>CK18 (P = 0.0077)</th>
<th>CK18/CREA (P = 0.0145)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (19)</td>
<td>119.3 ± 121.4</td>
<td>145.8 ± 189.3</td>
<td>13.4 ± 18.5</td>
<td>22.1 ± 36.1</td>
</tr>
<tr>
<td>12/19 (63.2)</td>
<td>10/19 (52.6)</td>
<td>7/19 (36.8)</td>
<td>7/19 (36.8)</td>
<td></td>
</tr>
<tr>
<td>2 (28)</td>
<td>314.1 ± 502.9</td>
<td>437.0 ± 789.3</td>
<td>19.9 ± 22.0</td>
<td>25.3 ± 37.6</td>
</tr>
<tr>
<td>19/28 (67.9)</td>
<td>16/28 (57.1)</td>
<td>18/28 (64.3)</td>
<td>17/28 (60.7)</td>
<td></td>
</tr>
<tr>
<td>3 (33)</td>
<td>388.0 ± 536.4</td>
<td>443.5 ± 650.7</td>
<td>47.1 ± 64.6</td>
<td>54.5 ± 79.6</td>
</tr>
<tr>
<td>31/33 (93.9)</td>
<td>28/33 (84.8)</td>
<td>28/33 (84.8)</td>
<td>27/33 (81.8)</td>
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</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Treatment</th>
<th>BTF</th>
<th>BTF/CREA</th>
<th>CK18</th>
<th>CK18/CREA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitomycin C</td>
<td>152/383 (39.7)</td>
<td>128/383 (33.4)</td>
<td>64/383 (83.3)</td>
<td>77/383 (20.1)</td>
</tr>
<tr>
<td>UTIs</td>
<td>43/152 (28.3)</td>
<td>43/152 (33.6)</td>
<td>8/152 (12.5)</td>
<td>10/152 (6.7)</td>
</tr>
<tr>
<td>First instillations</td>
<td>40/152 (26.3)</td>
<td>32/152 (25.0)</td>
<td>3/152 (18.7)</td>
<td>1/152 (6.3)</td>
</tr>
<tr>
<td>CREA &lt;60 mg/dl</td>
<td>16/152 (10.5)</td>
<td>10/152 (6.5)</td>
<td>3/152 (18.7)</td>
<td>1/152 (6.3)</td>
</tr>
<tr>
<td>Oncothiotepa</td>
<td>17/53 (32.1)</td>
<td>10/53 (18.9)</td>
<td>13/53 (24.5)</td>
<td>18/53 (34.0)</td>
</tr>
<tr>
<td>UTIs</td>
<td>5/17 (29.4)</td>
<td>4/10 (40.0)</td>
<td>4/10 (40.0)</td>
<td>4/10 (40.0)</td>
</tr>
<tr>
<td>First instillations</td>
<td>4/17 (23.5)</td>
<td>3/13 (23.1)</td>
<td>1/13 (7.7)</td>
<td>1/13 (7.7)</td>
</tr>
<tr>
<td>CREA &lt;60 mg/dl</td>
<td>3/17 (11.8)</td>
<td>3/10 (30.0)</td>
<td>2/13 (15.4)</td>
<td>7/18 (38.9)</td>
</tr>
<tr>
<td>BCG</td>
<td>50/86 (59.1)</td>
<td>32/86 (37.2)</td>
<td>16/86 (18.6)</td>
<td>17/86 (19.8)</td>
</tr>
<tr>
<td>UTIs</td>
<td>17/50 (34.0)</td>
<td>13/32 (40.6)</td>
<td>4/16 (25.0)</td>
<td>5/17 (29.4)</td>
</tr>
<tr>
<td>First instillations</td>
<td>45/50 (90.0)</td>
<td>30/32 (93.7)</td>
<td>8/16 (50.0)</td>
<td>9/17 (52.9)</td>
</tr>
<tr>
<td>CREA &lt;60 mg/dl</td>
<td>7/50 (14.0)</td>
<td>7/32 (21.9)</td>
<td>0/16 (0.0)</td>
<td>1/17 (5.9)</td>
</tr>
</tbody>
</table>

* UTIs, urinary tract infections.
could confound in the monitoring of patients with bladder and 21 for CK18/CREA). Sporadic elevated results for BTF observation was considerably greater for CK18 taking in con-
and 94 for CK18/CREA) during the serial monitoring. This negative results (50 for BTF, 57 for BTF/CREA, 87 for CK18, was that there was a considerable number of patients with true
observation during the disease with urinary tumor markers
and healthy subjects. Moreover, a statistically significant asso-
ciation with the most relevant clinical parameters of bladder
tumor marker depending on the differential benign or malignant
diseases studied is shown in Table 6. Urinary tract infection and
the presence of benign prostate hyperplasia were the most
frequent benign factors associated with elevated results for
CK18 and BTF tests in patients harboring urological diseases
other than bladder cancer. Both urinary tumor markers appeared
to be elevated in patients with prostate cancer.

**DISCUSSION**

Among the new molecules that might have a potential role
for the diagnosis of bladder cancer, urinary fibronectin and
CK18 represent feasible alternatives because both are present in
the bladder urothelium, might differentiate normal from bladder
tissue, and can be detected in the urine (10–27). Indeed, we
found higher urinary concentrations in patients harboring a
bladder tumor than in with those with no evidence of disease and healthy subjects. Moreover, a statistically significant asso-
ciation with the most relevant clinical parameters of bladder
cancer, stage and grade, was observed. At present, most of the
previous studies evaluating other urinary tumor markers were
cross-sectional, collecting urine samples once in the clinical
surveillance of patients with bladder cancer. They focused on
their potential diagnostic role as adjuncts or substitutes to con-
tventional detection methods (4–9) in selected groups of patients
and controls whose clinical status were confirmed by cystos-
copy, the gold standard. In addition to such a mandatory initial
approach in the evaluation of any tumor marker, we monitored
patients with bladder cancer with serial urinary tumor marker
measurement for 1 year to assess their role in the early detection
of any recurrence and to survey their clinical status, an approach
which, to our knowledge, no preceding study has described.

First of all, we focused on controlling the reliability of the
results through the analytical evaluation of the tests, the selection
of the most representative specimen, the endorsement of proper
sample handling and collection periods, or the explora-
tion of crucial factors, knowledge of which is essential for an
appropriate clinical interpretation of the results. The analytical
validation was favorable enough to support the introduction of
the test into the clinical daily practice from the laboratory point
of view. The lability and stability of fibronectin in the urine, extremely sensitive to proteolysis (21), justified the selection of
second micturition-voided urine samples for urinary marker
measurement rather than 24-h samples (45). Because we were
aware of the importance of appropriate sample handling, all
specimens were collected in the urological office and immedi-
ately centrifuged and frozen until measurement in the labora-
tory. We checked that the differential collection of urine sam-
ples before cystoscopy or before surgery in patients harboring a
bladder tumor could be a potential factor biasing diagnostic
characteristics. Intraindividual urine variations and the waiting
period between cystoscopy and surgery allowed tumor markers
to reach pathological cutoffs, increasing the sensitivities of
urinary tumor markers in preoperative urine samples.

The high variability of intraindividual urinary CREA con-
centrations justified why volumetric urinary tumor markers
might not always reflect the real clinical status. Although glo-
ally no statistical difference among CREA concentrations was
found in our series between volumetric and normalized ROC
curves as previously reported (8, 15, 17, 18), the correction of
urinary markers by CREA might balance the interindividual
urinary variations among populations. Urinary CREA concentra-
tions might differ among studies including different groups of
subjects or performed in different populations (8, 15, 46). There-
fore, normalized cutoffs are orientative, they are not transferable
among populations, and their recalculation appears to be rec-
ommended. Normalized units seem to be more difficult to deal
with; however, normalization appears to be essential in fol-

**Table 6**  False positive rates in benign and malignant urological diseases other than bladder cancer (group 4)

<table>
<thead>
<tr>
<th>Diagnosis (n)</th>
<th>BTF/CREA</th>
<th>CK18</th>
<th>CK18/CREA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benign diseases</td>
<td>11/21 (52.4)</td>
<td>7/21 (33.3)</td>
<td>6/21 (28.6)</td>
</tr>
<tr>
<td>Lithiasis (3)</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Stenosis (1)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>BPH* (13)</td>
<td>7</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>UTI (4)</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Malignant diseases</td>
<td>11/25 (44.0)</td>
<td>11/25 (44.0)</td>
<td>6/25 (24.0)</td>
</tr>
<tr>
<td>Kidney cancer (3)</td>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Prostate cancer (21)</td>
<td>9</td>
<td>9</td>
<td>6</td>
</tr>
</tbody>
</table>

*BPH, benign prostate hyperplasia; UTI, urinary tract infection.

**Specificity in Other Urological Conditions.** Taking volumetric cutoffs, overall false positive rates of urinary tumor markers in benign urological and other malignancies of different origin than the bladder were 22 of 45 (48.9%) for BTF and 12 of 45 (26.7%) for CK18 and when corrected by urinary CREA 18 of 45 (40.0%) for BTF of CREA and 12 of 45 (26.7%) for CK18/CREA. The distribution of false positive rates of each tumor marker depending on the differential benign or malignant
diseases studied is shown in Table 6. Urinary tract infection and
the presence of benign prostate hyperplasia were the most
frequent benign factors associated with elevated results for
CK18 and BTF tests in patients harboring urological diseases
other than bladder cancer. Both urinary tumor markers appeared
to be elevated in patients with prostate cancer.

**Volumetric cutoffs** were 52.8 mg/liter for BTF, 7.4 mg/liter for CK18, and 77 mg/liter for BTF/CREA; cutoff was 7 µg/g for CK18/CREA when normalizing by urinary CREA.

**DISCUSSION**

Among the new molecules that might have a potential role
for the diagnosis of bladder cancer, urinary fibronectin and
CK18 represent feasible alternatives because both are present in
the bladder urothelium, might differentiate normal from bladder
tissue, and can be detected in the urine (10–27). Indeed, we
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subjects or performed in different populations (8, 15, 46). There-
fore, normalized cutoffs are orientative, they are not transferable
among populations, and their recalculation appears to be rec-
ommended. Normalized units seem to be more difficult to deal
with; however, normalization appears to be essential in fol-
low-up studies for a better comparison of variations of urinary tumor markers within a patient whose concentrations may vary greatly due to shorter or longer micrurition periods rather than to increases or decreases in volumetric tumor markers. Consequently, the analysis of an individual chart showing the normalized variations of tumor markers through time should be considered before taking any clinical decision based on urinary tumor markers evolution.

Given the presence of fibronectin in blood (25–27) and because hematuria is a frequent diagnostic sign for bladder cancer (1–3), the presence of blood in the urine could be expected to interfere in BTF results. However, we did not find statistically higher concentrations in patients with gross hematuria as compared with those with microhematuria or absence of blood in the urine (data not shown).

It is difficult to compare the additional diagnostic value of urinary BTF and CK18 with other urinary tumor markers in the absence of data performed in the same samples and with a different monitoring study design. Contrasting different ROC curves reported in descriptive studies, our data suggest a slightly higher sensitivity for BTF involving a reduction of specificity than NMP22, BTF, or fibrinogen degradation products (4, 5, 7, 15). CK18 showed a slightly lower sensitivity than other cytokeratin markers such as UBC, CYFRA 21-1, or tissue polypeptide-specific antigen at similar specificities (6, 8, 15–18). Overall, the negative predictive value of BTF were relatively high as compared with other markers previously explored (4–9, 15–18). However, its specificity and positive predictive value were lower than for CK18 as a consequence of its higher rate of false positive results. The simultaneous determination of both tumor markers slightly increased the sensitivity and the negative predictive value of BTF and subsequently decreased the specificity, finally giving a more favorable overall accuracy for CK18. Interestingly, both urinary tumor markers provided better diagnostic accuracies than urinary cytology and the presence of micro- and/or macrohematuria. Cytology sensitivity was lower than for both urinary tumor markers accordingly to the considerable number of low grade and low stage tumors included in this study and the absence of carcinoma in situ in our series. These observations support the introduction of urinary tumor markers as potential substitutes for urinary cytology for the monitoring of the disease, keeping in mind the high specificity of cytology, which justify its use in current protocols (1, 3). Even the gold standard of bladder cancer does not always imply a 100% sensitivity.

Most false negatives for BTF were low risk patients in terms of stage and grade or were associated with low urine concentration, which prevents tumor markers from reaching cutoff levels without normalization. All invasive tumors were detected by BTF, an observation supporting the interaction between the tumor cells and the basement membrane contributing to the invasive phenotype by a higher ability of the cancer cell to produce proteolytic enzymes degrading basement membrane components such as fibronectin (35, 36, 47). On the contrary, the lower sensitivity of CK18 in a few invasive bladder cancers seems to be associated with the release of CKs in invasive disease directly into the blood rather than into the urine (48), whereas superficial disease appears to be easily detected in urine rather than in serum, an observation in accordance with low serological sensitivities already described with other CK markers (46, 48, 49). For both urinary markers, the Hook effect was discarded in those invasive tumors in which urinary tumor markers did not reach cutoffs (50).

False positive results were significantly higher for BTF than for CK18 and might lead to a clinical misinterpretation of the tests. Infections of the lower urinary tract and benign or malignant alterations of the prostate, common situations in patients with bladder cancer, were the most frequent factors associated with elevated tumor markers in urological patients free of bladder tumor (8, 15) and responsible for nearly one-third of elevated nonexpected results during monitoring of patients of groups 2 and 3. The additional performance of urinalysis before measurement of urinary tumor markers could help select appropriate samples for laboratory testing, so that those that hinted urinary tract infections could be excluded and a new one could be requested. Given the presence of CKs and fibronectin in the prostate (51, 52), benign prostatic hyperplasia or prostate cancer are to be discarded in order to avoid being misled to a diagnostic cystoscopy based on urinary tumor marker results. The main drawback for BTF was during the monitoring of intravesical therapy because of the frequent false positive results during the first weeks related to the chemical cystitis associated with mitomycin C, with Oncothiopeta, and most of all with BCG. Indeed, fibronectin appears to be essential for the intravesical binding of BCG to the bladder wall (53), although its role in human bladder urothelium has not been completely demonstrated (31). BTF preferably should not be measured before basal levels are reached after surgery, because the iatrogenic abrogation of the intact urothelial wall by resection, fulguration, and therapeutic manipulation might alter urinary fibronectin concentrations in patients with bladder neoplasms (31).

Urinary tumor markers are expected to help in those situations in which cystoscopies are necessary, and any other alternative noninvasive diagnostic method might provide information to individualize them. Screening patients with micro- and/or macrohematuria of unknown origin might greatly benefit from the information provided by urinary tumor markers to avoid uncomfortable and in some cases presumably unnecessary cystoscopies. For such purpose, their negative predictive value is essential to be high and reliable enough not to miss a tumor in addition to a high sensitivity. Both tumor markers showed a favorable profile in this situation. If a tumor marker is positive, they might help select those patients with micro- or gross hematuria who require immediate cystoscopy for screening purposes. When the biomarker is negative, the relative risk of missing a bladder cancer patient should be assumed if cystoscopy is not going to be performed, situation in which follow-up with urinary tumor markers appears to be recommended.

Follow-up of patients with prevalent disease is essential. BTF and CK18 could accelerate diagnosis before the scheduled cystoscopies in a considerable number of cases. More interestingly, persistent negative results in those patients submitted for follow-up with no evidence of disease were true negative results as revealed by scheduled cystoscopies. On the basis of the high negative predictive values we found, these patients would greatly benefit from urinary tumor marker monitoring and the number of cystoscopies could be substantially reduced in long
term follow-ups. Only studies monitoring serial urinary tumor markers can prove whether their reliability are high enough to increase the intervals between cystoscopies. Our data support consideration of serial urinary tumor markers as adjuncts, providing interesting orientative information to individualize the periods between cystoscopies.

Patients receiving intravesical chemo- or immunotherapy is a third related situation in which monitoring of the disease with urinary tumor markers could be beneficial. Similarly, the most relevant finding of this study was that persistent negative results of both tumor markers during monitoring were in agreement with those of the subsequent cystoscopies. Thus, intercystoscopy periods might have been increased, with a minimal risk of overlooking bladder tumors. CK18 showed the best profile in our series, the high false positive rates found for BTF associated with chemical cystitis related to intravesical instillations suggest that patients with urinary BTF receiving BCG not be monitored. Further studies would reveal whether this finding might imply a prognostic value. However, the three major factors associated with elevated results could be easily be controlled: (a) urinary tract infections through the performance of urinalysis before tumor marker measurement; (b) chemical cystitis associated with intravesical chemo- or immunotherapy by starting measuring urinary tumor markers once the basal level is reached after weekly instillations; and (c) changes in urine concentrations that might lead to higher elevated results than expected by analysis of individual charts for each patient normalizing by urinary CREA. Further work must be performed concerning clinical circumstances (such as other therapy) that may account for misinterpretation of the data. Although these were not the objectives of our study, there are insufficient data to suggest whether this urine marker is of value in anticipating more aggressive disease; i.e., there is no evidence to suggest that these markers are of value in anticipating virulent progression of cancer.

CK18 and BTF in urine may eventually prove to be of benefit for specific patients with bladder carcinoma given their higher sensitivity than that of cytology as alternative objective noninvasive diagnostic methods. Once the major sources leading to a clinical misinterpretation of the elevated results are controlled, their high predictive values provided enough reliability to monitor the status of the disease and presumably to individualize the intervals between cystoscopies. In selected patients, namely those with persistent negative urinary CK18 and BTF, the number of cystoscopies could be reduced. Monitoring of patients with bladder cancer with serial urinary tumor marker measurement was specially beneficial for follow-up patients with and without adjuvant intravesical chemo- or immunotherapy.

ACKNOWLEDGMENTS

We thank Diagnostic Products Corporation for donating reagents. We are also grateful to Ruth Ortiz, Carmen San Feliciano, Cefe Oterino, and Caridad Ruano for assistance in collecting patients’ samples.

REFERENCES


Evaluation of Two New Urinary Tumor Markers: Bladder Tumor Fibronectin and Cytokeratin 18 for the Diagnosis of Bladder Cancer

Marta Sánchez-Carbayo, Manuel Urrutia, Jose Manuel González de Buitrago, et al.


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