Reflex UroVysion Testing of Bladder Cancer Surveillance Patients With Equivocal or Negative Urine Cytology

A Prospective Study With Focus on the Natural History of Anticipatory Positive Findings

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Abstract

A proportion of patients under surveillance for recurrent bladder carcinoma with no immediate evidence of bladder tumor recurrence have positive multitarget fluorescence in situ hybridization (FISH; UroVysion, Vysis, Downers Grove, IL) results. The course of these “anticipatory positive” cases and the time to bladder tumor recurrence remains unknown. We followed up 250 patients with urine cytologic results, concurrent multitarget FISH, and cystoscopic examination for recurrent urothelial carcinoma. Of 81 cases (32.4%) with FISH-positive results, tumor recurrence developed in 60 (74.0%). Of 169 (67.6%) FISH-negative cases, recurrent urothelial carcinoma developed in 22 (13.0%). Of 211 patients (84.4%) with negative cystoscopic examination results, 56 (26.5%) had positive FISH results, and in 35 (62.5%) of these patients, recurrent urothelial carcinoma developed. Approximately 27% of patients under bladder carcinoma surveillance without immediate evidence of tumor recurrence will have a positive FISH result, defining the anticipatory positive subset. In about 65% of this anticipatory positive group, recurrent bladder urothelial carcinoma developed within 29 months.

Bladder cancer is the fourth most common type of cancer occurring in US men.1 Urothelial carcinoma (UC) of the bladder accounts for the majority of primary bladder cancers, with squamous cell carcinoma and adenocarcinoma accounting for fewer than 10%. In general, UC is grouped into 1 of 2 categories: high- or low-grade lesions based on their genetic profile and clinical behavior.2,3 The majority of bladder primary UCs are low-grade (grades I and II), noninvasive (pathologic stage pTa) papillary lesions and are typically treated with simple surgical excision. However, UC is associated with a high rate of recurrence (70%) and a distinct rate (10%-30%) of progression to high-grade lesions (grade III), carcinoma in situ, or invasive UC (pT1), which necessitate frequent patient surveillance4 or cystectomy (pT2 or higher).

The current reference standard for UC surveillance is cystoscopic examination in conjunction with urine cytologic examination every 3 months during the first 2 years with decreasing frequency thereafter.4,5 Limitations of the present surveillance algorithm include the need for frequent invasive evaluation of the bladder mucosa by cystoscopy, the relatively low sensitivity of urine cytology of around 34%,5 and the frequent occurrence of cytologically equivocal (“atypical”) results. This has led to the development of many adjunctive assays for the detection of UC recurrence, the goal of which is to identify patients for whom increased intensity of cystoscopic surveillance is a necessity while allowing safe extension of surveillance intervals for patients at minimal risk of recurrence. These adjunctive assays are urine-based immunoassays, such as BTA Stat (Polymedco, Cortlandt Manor, NY) and NMP-22 (Matritech, Newton, MA), or cell-based assays, such as ImmunoCyt (DiagnoCure, Quebec, Canada) and UroVysion (Abbott/Vysis, Downers Grove, IL).5-7
UroVysion is a multtarget fluorescence in situ hybridization (FISH) assay designed to detect chromosomal alterations associated with low- and high-grade UC lesions. It is composed of 3 DNA probes directed toward the pericentromeric regions of chromosomes 3, 7, and 17 and a fourth probe to the 9p21 locus. This combination of probes allows for the detection of chromosomal aneusomy and/or deletion of the 9p21 region (locus of the p16 gene), which are common genetic alterations occurring in UC.8,9 At our institution, exfoliated transitional cells from voided or instrumented urine collection specimens are first cytologically examined, and, if results are negative or atypical, the cells are “reflected” for UroVysion testing.10 Cytologically positive cases are not reflexed for FISH analysis because the specificity of urine cytologic examination alone approaches 100%5 and samples positive by cytologic definitions and FISH assay for all patients was 23 months (range, 12-30 months).

Each patient was then examined approximately every 3 months and/or subsequent cytologic diagnosis. The median follow-up such as a positive cystoscopic result, a positive surgical biopsy, or atypical urine cytologic results. Such cases have been termed “anticipatory positive.”15 However, the exact percentage of such anticipatory positive cases in which UC actually develops, the time interval at which they occur, and whether they are significant tumors altering the clinical management strategy remain largely unknown.

Materials and Methods

Study Population

The study population consisted of all bladder cancer surveillance patients with available clinical follow-up who had a negative or atypical urine cytologic diagnosis between June 2002 and December 31, 2003. At the time of the cytologic diagnosis, each patient within this population was reflexed for UroVysion FISH analysis and given a cystoscopic examination. Each patient was then examined approximately every 3 months thereafter until January 1, 2005, to detect signs of recurrent UC, such as a positive cystoscopic result, a positive surgical biopsy, and/or subsequent cytologic diagnosis. The median follow-up for all patients was 23 months (range, 12-30 months).

Cytologic Definitions and FISH Assay

All urine cytologic specimens were evaluated using a 4-tier classification system of unsatisfactory, negative, atypical or “suspicious,” and positive. Atypical and atypical or suspicious diagnoses were reserved for cases in which there were atypical groupings of urothelial cells with an increased nuclear/cytoplasmic ratio with mild to moderate nuclear enlargement and nuclear irregularities. These atypical cases represent specimens in which the differential diagnosis included reactive cellular changes vs low- or high-grade UC. Only cases with unequivocal cytologic findings of low- or high-grade UC were called positive.

The method for our direct FISH assay on ThinPrep-based (Cytyc, Marlborough, MA) specimens was previously published.12 Briefly, Papanicolaou-stained ThinPrep urine cytology slides were reviewed by a cytopathologist (J.A.B. or C.V.B.), and the areas containing the urothelial cells of interest were marked on the underside of the glass slide with a diamond-tipped pen. The coverslips and mounting medium were gently removed via xylene, rinsed, partially hydrated, and decolorized. The uncovered slides were incubated in a protease buffer at 37°C, fixed in 1% formaldehyde, and rehydrated. After the slide was completely air dried, 8 µL of the UroVysion probe set was applied, and the slide was immediately coverslipped and sealed with rubber cement. The cellular and probe set DNA are denatured at 73°C for 5 minutes in a HYBrite denaturation/hybridization system (Abbott/Vysis). Hybridization occurs in a prewarmed (37°C), humidified box overnight (12-18 hours). After hybridization, the rubber cement and coverslip were gently removed, and the slide was treated with a series of stringency washes (1× standard sodium citrate [SSC]/0.3% Nonidet P-40 [NP-40] for 2 minutes, followed by 2× SSC/0.1% NP-40 at room temperature for 1 minute, a quick dip in 2× SSC, and then distilled water). The slide was allowed to completely air dry in darkness, and 15 µL of Vectashield (Vector Laboratories, Burlingame, CA) with 4'-6-diamidino-2-phenylindole (DAPI) counterstain was added to the target area of the slide, coverslipped, and sealed with rubber cement.

UroVysion Interpretation

A minimum of 25 morphologically abnormal cells were visualized using the DAPI counterstain and initially evaluated for chromosomal changes. If no abnormalities were detected or no atypical cells were present on the slide, assessment of the remaining cells continued until a sufficient number of abnormal cells was found or until all cells were evaluated. In cases with no apparent abnormalities (eusomic for all 4 markers) but fewer than 25 cells available for analysis, a negative result was given with a limited cellularity comment added to the report. A positive case must contain cells with 1 of the 3 following criteria: (1) multiple chromosomal gains (>2) of chromosomes 3, 7, or 17 in at least 4 analyzed cells; (2) homozygous 9p21 deletion in at least 12 analyzed cells; or (3) isolated trisomy of chromosome 3, 7, or 17 in at least 10% of analyzed cells.
Outcomes and End Points

For each patient, the time at which the initial cytologic, FISH, and cystoscopic examinations were carried out was recorded as the patient’s starting point. A patient’s end point was defined as any evidence of UC recurrence by any concurrent or subsequent positive cystoscopic examination result or as biopsy- or cytologic-proven tumor recurrence. For all patients with an initial positive FISH assay result, results remained positive throughout the study. For all patients with an initial negative FISH assay result, results remained negative throughout the study unless any subsequent FISH assay result became positive before the defined end point (UC recurrence), as was the case for 2 patients in whom the initial starting point was reclassified accordingly owing to the possibility of early detection of UC recurrence by FISH. The time to UC recurrence from a FISH-positive or FISH-negative result was left truncated and interval censored for all patients in the study because the exact time of bladder recurrence could not be measured exactly but rather was only known to lie between 2 time points. For a patient in whom UC did not recur within the follow-up period, the recurrence time was right censored at the patient’s last follow-up.

Statistical Methods

Population characteristics were analyzed to detect imbalance in the cohort (older than 60 years, sex, and specimen type) via a 1-sample proportions test with continuity correction. Ignoring the left-truncated nature of the data, the probability of no recurrence after study entry was estimated for the patient subgroups by using a nonparametric maximum likelihood estimate, which produces estimates of survival probabilities for interval-censored data that are similar to Kaplan-Meier estimates. The probability of no recurrence for different groups was compared by using a log-rank statistic. Note that the log-rank statistic used herein can be applied only to right-censored data, so any interval-censored UC recurrence times were approximated by the midpoint of the interval. All statistical comparisons were based on a .05 significance level, and no method for accounting of multiple comparisons was made. Statistical analysis was carried out using the statistical software packages, SAS version 9.1 and R version 2.1.0 (SAS, Cary, NC).

Results

UroVysion FISH Detects Clinically Significant Lesions With Negative or Atypical Cytologic Diagnoses

In total, there were 812 UroVysion assays from 452 patients performed at our institution during the study period. Of the 452 patients identified 89 (19.7%) were lost to follow-up, or their samples were from our reference laboratory where clinical follow-up data were not available for review. Of the remaining 363 patients, 113 (31.1%) had no prior history of UC, whereas 250 (68.9%) were under recurrent UC surveillance protocols. The latter set of 250 patients formed our study population. Demographic features of this study population are given in Table 1, indicating that the majority (84.4%) of the 250 patients were older than 60 years (median, 72 years; range, 38-92 years), 74.8% were men, and the specimens for 77.6% of patients were voided.

Table 2 summarizes the cytologic, FISH, and cystoscopic diagnoses with outcome data. Recurrent UC developed in 32 (19.3%) of 166 cases with a negative cytologic diagnosis...
and 50 (60%) of 84 cases with an atypical cytologic diagnosis. In contrast, 60 (74%) of the 81 FISH-positive cases and 22 (13.0%) of 169 FISH-negative cases developed recurrent UC.

For the 21 patients with a positive FISH result but in whom recurrent UC did not develop, 14 (67%) were not receiving any treatment, 3 were receiving bacille Calmette-Guérin therapy, 3 had a history of upper-tract UC, and 1 had received pelvic radiation for prostatic adenocarcinoma.

**Most Tumors Undetected by UroVysion FISH Are Low-Grade, Noninvasive UCs, Most Forming a Visible Mass on Cystoscopy**

Table 3 summarizes patient clinical outcome (as defined by the 1973 UC classification system16) for each type of cystoscopic and FISH diagnosis. Of the 60 FISH-positive cases with UC recurrence, 15 were low-grade UC and 44 were high-grade UC; 1 patient with a history of UC had newly diagnosed prostatic adenocarcinoma involving the bladder. Of the 22 FISH-negative cases with UC recurrence, 17 (77%) were low-grade UC and 5 (23%) were high-grade UC. Of the 17 low-grade lesions, 11 were visible by cystoscopy. The remaining 6 low-grade lesions not immediately apparent by cystoscopy became visible by cystoscopy within 3 to 10 months.

Of the 5 high-grade UC lesions with negative FISH results, only 2 were not visible by concurrent cystoscopy. The first case was a noninvasive, high-grade lesion in a patient with a history of UC involving the left renal pelvis who had undergone radical nephroureterectomy and had biopsy-documented noninvasive UC recurrences in the bladder. The second case was a muscle-invasive (pT2) UC of the “nested” type, histologically showing an intact benign overlying urothelium, in a patient with a history of superficially invasive (pTa) UC who had undergone resection and bacille Calmette-Guérin therapy. The remaining 3 high-grade, FISH-negative UC lesions were detected by cystoscopy and consisted of 1 noninvasive (pTa) lesion and 2 invasive (pT2) lesions. Of the latter 2 cases, 1 was a recurrent tumor involving the lower ureter and causing complete ureter obstruction. The second invasive UC case was a high-grade UC in the dome of the bladder for which a subsequent positive FISH result was found in an instrumented urine sample obtained before cystoscopy.

**Anticipatory Positive Cases Likely to Develop Recurrent UC Within 6 to 10 Months of a Positive FISH Result**

There were 56 patients with a negative concurrent cystoscopic examination result, negative or atypical cytologic diagnosis, and positive FISH result, defining the anticipatory positive subset. Of these 56 cases, 35 (63%) developed recurrent UC within the follow-up period. Of these recurrent tumors, 22 were classified as high-grade UC, 12 as low-grade UC, and 1 as a newly diagnosed prostatic adenocarcinoma. In contrast, recurrent UC developed in only 8 (5.2%) of 155 cystoscopically negative, cytologically negative or atypical, and FISH-negative cases.

**Summary of UC Recurrence by the 1973 Classification for Starting Cytologic, FISH, and Cystoscopic Diagnoses in 82 Cases**

<table>
<thead>
<tr>
<th>Low-Grade Lesions</th>
<th>High-Grade Lesions</th>
</tr>
</thead>
<tbody>
<tr>
<td>pTa</td>
<td>pTa</td>
</tr>
<tr>
<td>GI</td>
<td>GI</td>
</tr>
<tr>
<td>Negative FISH, negative cystoscopy (n = 8)</td>
<td>4</td>
</tr>
<tr>
<td>Negative FISH, positive cystoscopy (n = 14)</td>
<td>9</td>
</tr>
<tr>
<td>Positive FISH, negative cystoscopy (n = 35)</td>
<td>6</td>
</tr>
<tr>
<td>Positive FISH, positive cystoscopy (n = 25)</td>
<td>0</td>
</tr>
</tbody>
</table>

CISH, carcinoma in situ; FISH, fluorescence in situ hybridization; GI, grade I; GII, grade II; GIII, grade III; pT, pathologic tumor size in which a denotes a noninvasive lesion, 1 a superficially invasive lesion, and 2 a deeply invasive lesion; UC, urothelial carcinoma.

Newly diagnosed prostatic adenocarcinoma.
FISH result. However, this same estimate also showed a 48% chance (95% CI, 34%-62%) and 54% chance (95% CI, 40%-67%) of developing recurrent UC within 6 and 10 months of the positive FISH result, respectively.

**Discussion**

The data presented herein show that performing reflex UroVysion FISH analysis on ThinPrep-based, routine urine cytologic specimens allows early detection of recurrent UC in patients with a negative cystoscopic and cytologic diagnosis. Approximately 26% of patients with negative cystoscopic results will have a positive concurrent UroVysion FISH result, defining the anticipatory positive subset. According to the data presented herein, recurrent UC will develop in approximately 50% to 80% of this anticipatory positive population within 29 months of a positive FISH result. Although this 29-month period seems rather long, we also estimated that in 34% to 62% and 40% to 67% of the anticipatory positive population, recurrent UC developed within 6 and 10 months, respectively, of the positive FISH result.

**Table 4**

<table>
<thead>
<tr>
<th>Group</th>
<th>Recurrence (%)</th>
<th>95% CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total population</td>
<td>33 at 29 mo</td>
<td>27-39</td>
<td>—</td>
</tr>
<tr>
<td>FISH Negative</td>
<td>13 at 29 mo</td>
<td>8-18</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Positive</td>
<td>76 at 29 mo</td>
<td>65-87</td>
<td>—</td>
</tr>
<tr>
<td>Cytology</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atypical</td>
<td>61 at 29 mo</td>
<td>47-75</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Negative</td>
<td>19 at 29 mo</td>
<td>13-25</td>
<td>—</td>
</tr>
<tr>
<td>Negative cytology</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative FISH</td>
<td>12 at 29 mo</td>
<td>6-17</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Positive FISH</td>
<td>52 at 29 mo</td>
<td>34-69</td>
<td>—</td>
</tr>
<tr>
<td>Atypical cytology</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative FISH</td>
<td>18 at 20 mo</td>
<td>5-30</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Positive FISH</td>
<td>89 at 20 mo</td>
<td>78-100</td>
<td>—</td>
</tr>
<tr>
<td>Negative cystoscopy</td>
<td>20 at 29 mo</td>
<td>15-27</td>
<td>—</td>
</tr>
<tr>
<td>Negative FISH</td>
<td>5 at 29 mo</td>
<td>2-8</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Positive FISH</td>
<td>65 at 29 mo</td>
<td>50-80</td>
<td>—</td>
</tr>
</tbody>
</table>

CI, confidence interval; FISH, fluorescence in situ hybridization.
Previous retrospective studies performed in our laboratory showed UroVysion FISH to have an overall sensitivity of 85% and specificity of 97% for detection of recurrent UC. Other studies have reported that the sensitivity of FISH is higher, 94% to 100%, for high-grade urothelial lesions compared with low-grade urothelial lesions, for which the reported sensitivity ranges from 36% to 86%. This pattern is shown herein—the majority (11/14 [79%]) of the FISH false-negative cases that occurred were low-grade, noninvasive urothelial lesions. The fact that all low-grade, FISH false-negative lesions in this study remained FISH-negative, including at the time of surgical excision, supports the notion that many low-grade lesions do not actively shed neoplastic cells into the bladder lumen or, alternatively, such low-grade lesions do not exhibit the chromosomal changes that are detected by UroVysion FISH testing. Such cases will be the subject of future studies in our laboratory to provide an explanation for their current undetectability.

As measured by the proportion of positive cystoscopic results in cases that also had positive FISH results at the starting point, the sensitivity of UroVysion for the detection of recurrent UC within this patient cohort is estimated to be 64% (25/39; 95% CI, 47%-78%). Although this value is somewhat low, it lies within the spectrum of previously published values. It must be emphasized that the population in this study consisted only of negative or atypical cytology cases. During the 18-month enrollment time, there were 198 cytologic specimens from 134 patients signed out as “positive for malignant cells, urothelial carcinoma.” Previously published studies have established that the majority of cytologically positive cases are FISH-positive. If we include these 134 cases as FISH-positive cases, the estimated sensitivity of UroVysion FISH would be 91% (95% CI, 87%-95%). However, not performing FISH on these 198 cytologic specimens saved an estimated $86,000 in patient expenses per year, which had been the principal reason for developing and instituting the reflex testing approach on only negative or atypical cytology specimens as opposed to the testing of all patients in our laboratory.

There are 2 major limitations to this study. First, physicians were not blinded to any test results because the FISH test was done immediately after the cytologic test on the same slide and the physician had access to cytologic and FISH results when interpreting results of the cystoscopy. This undoubtedly influenced their decision making in the interpretation of test results. However, the primary goal of this study was to demonstrate how UroVysion FISH testing could be used to augment cystoscopic and cytologic examination to increase early detection of recurrent UC. This study was not designed to accurately calculate the sensitivity and specificity of UroVysion FISH because those studies have already been performed and, incidentally, results are similar to those estimated herein.

A second limitation is that patient enrollment occurred based on the first time UroVysion FISH was performed for any given patient rather than when a patient first became clinically disease-free. This led to left truncation of recurrence times that could not be accurately accounted for in the statistical analysis of recurrence times. Ignoring left truncation results in biased estimates of the probability of recurrence. Unfortunately, this limitation was unavoidable because the novelty of this assay prevented better time-controlled patient enrollment. Further studies with better controlled patient enrollment are underway at our institution.

There were 23 invasive (pT1 or pT2) UCs in this study. Of these 23 cases, FISH detected 20 (87%). Review of the 3 missed deeply invasive UC surgically resected specimens revealed that although all 3 lesions contained diagnostic molecular changes of UC detectable by UroVysion FISH, no diagnostic carcinoma cells were present in the initial urine cytologic preparations. This was explained in 2 of the 3 cases by the presence of an intact nonneoplastic surface urothelium overlying the nested carcinoma and by complete obstruction of urine flow in the distal ureter carcinoma. Consequently, neither of these 2 lesions was capable of shedding cancer cells into the bladder lumen, and, therefore, the carcinoma cells could not be detected in urine samples from these 2 patients. The third FISH-negative invasive tumor was a high-grade UC situated in the dome of the bladder. Although the originally tested voided urine specimen failed to show chromosomal abnormalities, a subsequently obtained instrumented urine sample showed positive FISH findings. These 3 cases further display the common limitation of urine cytologic examination and UroVysion FISH—cancer cells need to be physically present in the urine samples for these cell-based assays to detect their presence.

Worthy of mention is that although UroVysion was initially approved by the Food and Drug Administration (FDA) only for bladder tumor surveillance, it was recently granted FDA approval to be used as a bladder cancer screening tool in patients with hematuria. There were 113 such patients identified during this study who were excluded because the focus was on surveillance patients only. These 113 patients had no history of UC but did undergo UroVysion FISH owing to various complaints such as hematuria, painful urination, urinary incontinence, and/or frequency. Of these patients, 31 had FISH-positive results; of the 31, 17 had newly diagnosed bladder carcinoma. The estimated sensitivity and specificity of UroVysion FISH in this screening setting were 62% (95% CI, 32%-85%) and 77% (95% CI, 67%-85%), respectively, which is similar to the 67% to 68% sensitivity and 78% to 80% specificity reported in the multi-institutional trial leading to the FDA approval of this assay as a hematuria screening tool.

Another notable finding is that a positive UroVysion FISH result can be observed with non-UCs. In our experience, we have observed positive UroVysion FISH results in 2 bladder
primary adenocarcinomas, 2 bladder primary small cell carcinomas, 1 bladder primary squamous cell carcinoma, 1 rectal adenocarcinoma invading the urinary tract, and 1 renal cell carcinoma that invaded the renal collecting system. Although no controlled studies have been performed, this observation suggests that UroVysion could detect other carcinomas that can shed malignant cells into urine. In fact, aneusomy of chromosomes 3, 7, and/or 17 has been reported in other histologic types of bladder cancer,20 prostatic adenocarcinoma,21 colonic adenocarcinoma,22 and renal cell carcinoma.23

UroVysion FISH is an excellent adjunct to ThinPrep-based urine cytology, with the capacity to detect recurrent UC before cystoscopically visible lesions can be identified and to resolve equivocal cytologic findings. Furthermore, 26% of cystoscopically negative patients under surveillance for recurrent UC had a positive UroVysion FISH result, and in approximately 65% of these patients, recurrent UC developed within 29 months. However, caution in relying on negative FISH results must be exercised in patients with involvement of the upper urogenital tract and with certain rare variants of UC that may not shed diagnostic tumor cells and, therefore, may escape detection.

References