The Development of a Multitarget, Multicolor Fluorescence in Situ Hybridization Assay for the Detection of Urothelial Carcinoma in Urine

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The purpose of this study was to develop a multitarget, multicolor fluorescence in situ hybridization (FISH) assay for the detection of urothelial carcinoma (UC) in urine specimens. Urinary cells obtained from voided urine specimens of 21 patients with UC and 9 normal donors were analyzed with nine different centromere enumeration probes and a single locus-specific indicator probe to determine an optimal set of FISH probes for UC detection. The four probes with the greatest sensitivity for UC detection were then labeled with a unique fluorophore and combined into a single probe set. The probes with the greatest combined sensitivity for UC detection were CEP3, CEP7, CEP17, and the 9p21 (P16) LSI. This probe set was used to evaluate urine specimens acquired from 179 patients for prospective testing (46 with biopsy-proven UC). FISH slides were evaluated by scanning the slide for cells with nuclear features suggestive of malignancy and assessing the FISH signal pattern of these cells for polysomy (ie, gains of two or more different chromosomes). A receiver operator characteristic curve revealed that a cutoff of 5 cells with polysomy as the positive criterion for cancer resulted in an overall sensitivity of 84.2% for patients with biopsy-proven UC and a specificity of 91.8% among patients with genitourinary disorders but no evidence of UC. This study demonstrates that a multitarget, multicolor FISH assay containing centromeric probes to chromosomes 3, 7, and 17 and a locus-specific probe to band 9p21 has high sensitivity and specificity for the detection of UC in voided urine specimens. (J Mol Diag 2000, 2:116–123)

Tumors arising from the urothelial mucosa that lines the urinary bladder, ureters, and renal pelvis are known as urothelial carcinoma (UC) and are among the most common malignancies of the bladder and upper urinary tract (ie, ureters and renal pelvis). Most cases of UC arise in the bladder, but renal pelvic and ureteral UC account for approximately 5% of UC.1 In the bladder, non-invasive papillary UC, UC in situ, and UC that has invaded not deeper than the lamina propria are referred to as superficial bladder cancer. UC that has invaded into the muscularis propria of the bladder is referred to as muscle-invasive bladder cancer. Patients with muscle-invasive disease generally undergo cystectomy in an attempt to prevent the development of metastatic bladder cancer. Patients with superficial bladder cancer, on the other hand, are followed at regular intervals (eg, every 3 months) for tumor recurrence and progression, usually with cystoscopy and cytology. Numerous studies have demonstrated that cytology has high specificity but poor sensitivity for bladder cancer detection.12–11 In addition to cytology, flow cytometry is widely used for the detection of UC. However, assays with higher sensitivity for detection of recurrent tumor in superficial bladder cancer patients are needed, because false negative test results place a patient at risk of progression to potentially fatal muscle-invasive bladder cancer.

Our understanding of the genetic changes that accompany urothelial tumor carcinoma initiation and progression are increasingly being elucidated.12–14 Homozygous deletions of the P16 gene at 9p21 are one of the most common alterations in UC and occur early in the development of both papillary UC and UC in situ.15–21 UC progression is accompanied by increased chromosomal instability and aneuploidy.12,22–25 Cytogenetic studies reveal frequent alterations of a variety of chromosomes including chromosomes 9, 17, 7, 11, 1, and others.12 These chromosomal alterations can be detected with fluorescence in situ hybridization (FISH).26–32 FISH utilizes fluorescently labeled DNA probes to chromosomal centromeres or unique loci to detect cells with numerical or structural abnormalities indicative of malignancy.

Previous investigators have demonstrated that FISH can be used to detect UC in voided urine or bladder washing specimens.36–32 However, despite its apparent promise for this use, FISH has not been used clinically to detect UC. Possible explanations for the slow introduc-

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tion of FISH for UC detection include (i) previous studies have generally used single FISH probes for their analyses, which may have limited the sensitivity and specificity of the assay, and (ii) the FISH procedures that have been used to evaluate urine specimens for evidence of UC have been too time-consuming to be clinically practical. The goal of this study was to develop a FISH assay for the detection of UC in urine specimens that can be used in clinical laboratories to monitor patients with superficial UC and possibly to screen high risk patients (eg, smokers) for UC. In this paper we have determined (i) which FISH probes (of a set of eight) have the highest sensitivity for UC detection, (ii) the sensitivity and specificity of a multicolor, multitarget FISH probe set containing the four most sensitive of these eight probes for UC in prospectively obtained urine specimens from patients under evaluation for bladder cancer, (iii) the methodology that should be used to evaluate slides for malignant cells, and (iv) the diagnostic criteria that should be used to consider a case positive for UC.

Materials and Methods

Patients

Urine specimens from 21 patients with UC and 9 normal donors were used to determine an optimal set of FISH probes for UC detection. The optimal probe set was then used to evaluate prospectively acquired urine specimens from 179 patients, 93 with and 86 without a history of UC. The 86 patients without UC were being evaluated for a variety of genitourinary symptoms and signs, including obstructive or irritating voiding symptoms, microhematuria, and incontinence. The voided urine specimens from the 179 patients were obtained immediately before cystoscopy for FISH analysis.

Urine Processing

Specimens for FISH were processed within 24 hours. Cells from voided urine were sedimented at 1200 × g for 8 minutes. The cell pellet was resuspended in 15 ml hypotonic solution (0.75 mol/L KCl) for 10 minutes. The cells were then sedimented again at 1200 × g for 8 minutes and resuspended in 10 ml of a 3:1 solution of methanol:glacial acetic acid. This was repeated two more times, and the final cell pellet was generally resuspended in 50 to 200 μl (depending on cell pellet size) of residual 3:1 methanol:acetic acid.

Slide Preparation

For the probe selection portion of the study, slides were prepared by dropping a portion of the cell pellet suspension onto standard glass slides with a Pasteur pipette and visualizing the cellularity of the slide with a phase contrast microscope. Additional cell suspension was dropped onto the slide, if necessary, until an appropriate cellularity had been reached. We subsequently found that the cells could be confined to a smaller area of the slide with the use of 12-well, 0.6-cm Shandon-Lipshaw slides (Shandon Inc., Pittsburgh, PA). By confining the cells to a smaller area of the slide, we decreased the amount of time that was required to analyze the cells by microscopy after FISH hybridization had been performed. Thus, for the larger study of 179 patients, slides were prepared by placing 3 μl, 10 μl, and 30 μl of cell suspension into three separate wells. The use of 3 μl, 10 μl, and 30 μl of cell suspension ensured that most samples had at least one circle with the appropriate density of cells. If the density of the cells was too high or too low, additional dilution or concentration of the cell suspension was performed and applied to a fourth well.

Probe Sets Used to Determine Optimal Probe Set

Probes to the pericentromeric regions of chromosomes 3, 7, 8, 9, 11, 15, 17, 18, and Y and to the 9p21 band were examined for their relative sensitivities at detecting UC in urine specimens. The choice of these probes was based on a review of the literature that revealed that numerical alterations of these chromosomes or chromosomal loci are among the most frequent chromosomal alterations in UC.12,15–25 Directly labeled fluorescent probes to the pericentromeric regions of chromosomes (CEP probes) and the probe to the 9p21 band (LSI 9p21) were purchased from Vysis (Downers Grove, IL). These labeled probes were then combined into one of three probe sets. The probes contained in each of these probe sets and the fluorophore used to label each probe are shown in Table 1.

Fluorescence in Situ Hybridization

FISH was performed in the following way. Slides were incubated in 2× standard saline citrate (SSC) at 37°C for 10 minutes, 0.5 mg/ml pepsin (pH 2.0) at 37°C for 13 minutes, phosphate buffered saline (PBS) at room temperature for 5 minutes, 1% formaldehyde at room temperature for 5 minutes, and PBS at room temperature for 5 minutes. The slides were then placed in 70%, 85%, and 100% ethanol for 1 minute each. The slides were then air-dried and 3 μl of cell suspension was denatured in 70%, 85%, and 100% ethanol for 1 minute each. The slides were then air-dried and 3 μl of the denatured probe were placed in each of the 3 wells containing specimen. The slide was then coverslipped, sealed with rubber cement, and incubated at 37°C overnight in a humidified chamber. The slides were then

<table>
<thead>
<tr>
<th>Table 1. Probe Sets</th>
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<tbody>
<tr>
<td>Probe set</td>
</tr>
<tr>
<td>A</td>
</tr>
<tr>
<td>B</td>
</tr>
<tr>
<td>C</td>
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</table>
washed in 0.4× SSC/0.3% NP-40 at 73°C for 2 minutes and rinsed in 2× SSC/0.1% NP-40. Three microliters of DAPI II counterstain were placed on the slide and cover-slipped.

### Results

#### Normal Value Study

To determine the criteria for abnormal probe copy number, we first determined the distribution of centromeric and locus-specific probe copy numbers in urinary cells from normal donors (Table 2). This was done by enumerating the FISH signals for each of the probes in probe sets A, B, and C in 100 consecutive cells of voided urine specimens from 9 normal donors. The CEP15 and CEP Y

### Table 2. Normal Value Study for Centromeric and Locus-Specific Probes

<table>
<thead>
<tr>
<th>Probe</th>
<th>Mean percentage of cells with signal copy number ± SD</th>
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<tbody>
<tr>
<td></td>
<td>0 signal</td>
</tr>
<tr>
<td>CEP 3</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>CEP 7</td>
<td>0.6 ± 1.7</td>
</tr>
<tr>
<td>CEP 8</td>
<td>0.1 ± 0.3</td>
</tr>
<tr>
<td>CEP 9</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>CEP 11</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>CEP 17</td>
<td>1.5 ± 3.9</td>
</tr>
<tr>
<td>CEP 18</td>
<td>0.6 ± 1.2</td>
</tr>
<tr>
<td>9p21</td>
<td>4.5 ± 6.3</td>
</tr>
</tbody>
</table>

Average percentage of cells with 0, 1, 2, 3, 4, or 5 or more signals in 100 consecutive cells from nine urine specimens from normal donors.

#### Table 3. Sensitivity of Individual Probes and Complementation Analysis for Urothelial Carcinoma Detection

<table>
<thead>
<tr>
<th>Patient</th>
<th>Tumor stage†</th>
<th>CEP3</th>
<th>CEP7</th>
<th>CEP8</th>
<th>CEP9</th>
<th>CEP11</th>
<th>CEP17</th>
<th>CEP18</th>
<th>LSI 9p21</th>
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<tr>
<td></td>
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<td>7.8%</td>
<td>6.5%</td>
<td>7.1%</td>
<td>7.1%</td>
<td>7.1%</td>
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<td>7.0%</td>
<td>16.9%‡</td>
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<tr>
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<td>ND</td>
<td>39</td>
<td>ND</td>
<td>39</td>
<td>ND</td>
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<td>66</td>
<td>pT3</td>
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<td>66</td>
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<td>5</td>
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<td>84</td>
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<td>0</td>
<td>2</td>
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<td>0</td>
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<td>18</td>
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<td>9</td>
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<td>24</td>
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<td>74</td>
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<tr>
<td>225</td>
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<td>44</td>
<td>69</td>
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<td>32</td>
<td>0</td>
<td>23</td>
<td>5</td>
<td>18</td>
<td>1</td>
</tr>
</tbody>
</table>

Sensitivity of probe: 14/19 (73.7%), 16/21 (76.2%), 11/19 (57.9%), 11/21 (52.4%), 10/19 (52.6%), 13/21 (61.9%), 8/19 (42.1%), 6/21 (28.6%)

Dark shaded cells indicate cases positive for malignancy with the CEP7 probe; grey shaded cells indicate cases negative for malignancy with the CEP7 probe but positive with other probes (i.e., CEP3, CEP7, or LSI 9p21).

†TNM tumor stage based on biopsy or surgical resection.

‡The cutoff for abnormal percentage of cells with tetrasomy is indicated below each probe except LSI 9p21.

§The cutoff for abnormal percentage of cells with homozygous deletion indicated below probe LSI 9p21.

*Positive cytology (grade 3 TCC) but no biopsy. However, imaging studies reveal an obvious renal pelvic tumor.

**Suspicious cytology (atypical cells suspicious for carcinoma) and previous pTa tumor.

***Negative cytology and biopsy, but previous biopsy revealed a grade 2 pTa tumor.

ND, not determined.
probe signal intensities were weak, and we were unable to collect data for these two probes. Monosomy rates for the centromeric probes were relatively high and ranged from 7.8 to 14.4% (Table 2). Trisomy and tetrasyom rates for the centromeric probes were lower and ranged from 1.0 to 3.0% and 0.4 to 1.5%, respectively. The frequency of hemizygous and homozygous 9p21 loss was 10.3 and 4.5%, respectively. The presence of normal multinucleated umbrella cells did not impact the observed polysony rates because the individual nuclei of the umbrella cells were disomic and distinct from one another by 1-4, phenylenediamine (DAPI) stain.

Sensitivity for UC of Individual Probes

Tetrasomy or greater was much less frequent than trisomy in normal donor urines (Table 2). On the other hand, tetrasyom or greater was a frequent finding in cells from UC patients (Table 3). This suggested that the finding of tetrasyom or greater cells in urine specimens might be more indicative of UC than the finding of trisomic cells. For this reason, we used tetrasyom or greater as our criterion for evidence of FISH abnormality with individual probes in the patients with UC. A specimen was considered positive for cancer if the percentage of cells with tetrasyom or greater for a centromeric probe was greater than or equal to the mean number of cells with 3 SD with tetrasyom or greater observed in normal donors. The percentage of urine specimens from the 21 patients with UC that were FISH-positive by this criterion for each centromeric probe is shown at the bottom of Table 3. The sensitivity of tetrasyom of the centromeric probes for UC ranged from 42.1% for CEP18 to 76.2% for CEP 7.

For the 9p21 locus-specific probe, we focused on homozygous deletion of 9p21 as the criterion for positivity due to the relatively low frequency of this event in normal cell populations when compared to hemizygous 9p21 deletion (Table 2) and because it has been reported to be a frequent alteration in early stage UC.15–21 The sensitivity of homozygous 9p21 deletion for UC in the 21 UC cases was 28.6% (Table 3). Additionally, we also determined the sensitivity of chromosome 9 monosomy (ie, single copy of CEP 9 probe) for UC in the 21 UC cases (data not shown in Table 3), since this has also been shown to be a frequent event in UC.15–21 The sensitivity of chromosome 9 monosomy in the 21 UC cases, using a cutoff of 30.8% (mean percentage in normals + 3 SD), was 1/21 (4.8%).

Complementation Analysis to Determine the Combination of Probes with the Greatest Sensitivity for UC

Complementation analysis was performed to determine the combination of probes that provides the greatest overall sensitivity for UC detection (Table 3). Complementation analysis is important because a probe might have relatively low sensitivity for UC when used alone but might nonetheless enhance the overall sensitivity of the assay by complementing other probes. As noted above, the CEP7 probe was the most sensitive, having detected 16 of the 21 patients (darkly shaded cells in Table 3). The CEP3 and CEP17 probes complemented the CEP7 probe by detecting abnormalities in two and one additional patients, respectively, who did not show abnormalities with the CEP7 probe (lightly shaded cells in Table 3). Additionally, the 9p21 locus-specific probe detected two more UC cases (patients 223 and 239) that were not detected with any of the centromeric probes (lightly shaded cells in Table 3). Thus, with this group of UC patients, the highest sensitivity of UC detection was achieved with probes to the centromeres of chromosomes 3, 7, and 17 and a probe to the 9p21 band. Together these probes detected 20 of the 21 (95.2%) UC cases. For these same 21 UC cases, urine cytology was positive, equivocal, and negative for 13, 3, and 5 patients, respectively. If equivocal cytology results are scored as positive, the sensitivity of cytology for this set of patients was 76%.

Based on the results of the probe selection study we concluded that a probe set containing the CEP7, CEP3, CEP17, and the LSI probe 9p21 would have the greatest sensitivity for UC detection. The CEP7, CEP3, CEP17, and LSI 9p21 probes were labeled with Spectrum Green, Spectrum Red, Spectrum Aqua, and Spectrum Gold, respectively, and combined into a single probe set. This probe set was then used to prospectively analyze 181 urine samples from 179 patients with a history of UC or other genitourinary signs and symptoms. The goals were further refinement of the criteria that should be used to determine whether a case is positive or negative for cancer and determination of the sensitivity and specificity of FISH based on these criteria.

Development of Scanning Method for Evaluation of FISH Slides for UC

Our experience with FISH from patients with biopsy-proven UC and positive urine cytology findings has revealed that cells with FISH abnormalities suggestive of malignancy (eg, gains of multiple chromosomes) generally exhibit nuclear abnormalities. An example of the nuclear abnormalities observed in cells that are also abnormal by FISH is shown in Figure 1. The nuclear abnormalities observed include nuclear enlargement, irregular nuclear borders, and patchy (ie, nonhomogeneous) DAPI staining. We have also noted that polysomy (ie, gains of two or more different chromosomes in the same cell) is a more specific indicator that a cell is malignant than the gain of a single chromosome. Gains of a single chromosome appear more often to be artifactual, perhaps resulting from factors such as signal splitting or cross-hybridization. Based on these observations, we concluded that the most efficient way to evaluate slides
for cells with FISH abnormalities is to scan for cells with nuclear abnormalities suggestive of malignancy and to determine the CEP and LSI signal copy numbers in the suspicious cells.

The scanning method that we used is similar to the technique that cytopathologists and cytotechnologists use when they screen cytology slides. With both cytology and the FISH scanning method, the viewer scans the slides for cells with morphological features suggestive of malignancy and largely ignores the inflammatory cells (eg, neutrophils) and normal epithelial cells (eg, squamous or transitional cells). The main difference between cytology and FISH is that the morphologically abnormal cells are classified as malignant by FISH if they demonstrate chromosomal abnormalities and classified as malignant by cytology if the cells show morphological features such as nuclear hyperchromasia and nuclear irregularity.

For the portion of the study that used scanning, a cell was considered abnormal if it was polysomic (ie, showed gains of multiple chromosomes). Representative examples of normal and polysomic cells are shown in Figure 2. For the purpose of data collection and standardization of the scanning technique, the slide was scanned for 20 morphologically abnormal cells and the FISH signal pattern in those cells recorded.
Sensitivity of Scanning Method versus Counting Method

The relative sensitivities of evaluating consecutive cells for FISH abnormalities (counting method) versus the scanning method (described above) for UC were determined for 30 patients with biopsy-proven UC and 10 normal donors. Urine specimens were hybridized with the optimal probe set (ie, CEP3, CEP7, CEP17, and LSI p21) and evaluated with both the scanning and counting methods. The counting method was performed by enumerating the number of copies of each FISH probe in 100 consecutive cells. A case was considered positive for UC by counting if ≥7.6% (the mean percentage + 3 SD of cells with polysomy observed in specimens from normal donors) of the cells demonstrated polysomy and positive for UC by scanning if ≥4 of the 20 cells (the mean percentage + 3 SD of cells with polysomy observed in specimens from normal donors) demonstrated polysomy. The specificity of the two approaches was comparable: 93.2% for counting method versus 88.4% for the scanning method (P = 0.46). However, the sensitivity of scanning method was significantly better than for the counting method (86.2% versus 62.0%, respectively; P = 0.04).

Further Refinement of the Criteria for Considering a Case Positive for UC

We then used the CEP3, CEP7, CEP17, LSI 9p21 probe set and the scanning method to evaluate prospectively 181 urine specimens from 179 patients and 43 normal donors. Patient samples were evaluated without knowledge of the patient’s history, clinical or biopsy findings. Ninety-three patients had a previous history of bladder cancer and 86 did not. Biopsies or surgical resections were performed on 74 of the 179 patients. The surgical pathology results for these 74 patients were classified as positive for UC in 46 patients. Among these 46 tumors were 22 pTa tumors, 12 pTis tumors, and 12 pT1-pT4 tumors. The tumor grade for the 46 tumors was grade 1 for 8 tumors, grade 2 for 14 tumors, and grade 3 for 22 tumors. Although rare polysomic cells could be found in the urine of normal donors, the number of cells with polysomy never exceeded 5 cells for any of the 43 normal donors. Additionally, only 8.2% of the patients without evidence of UC (63 of the 179 patients) but with other genitourinary symptoms and signs had more than 5 cells with polysomy. Of the patients with biopsy-proven UC (n = 46), 84.2% had five or more cells with polysomy.

A receiver-operator characteristic curve was generated to determine the effect of various cutoffs on the sensitivity and specificity of the assay (Figure 3). The sensitivity and specificity of the assay for UC was 89.5% and 87.8%, respectively, if 4 or more cells with polysomy was used as a cutoff for positivity and 84.2% and 91.8%, respectively, using a cutoff of 5 or more cells with polysomy for positivity (Figure 3).

The majority of the false negative FISH results among patients with biopsy-proven UC were obtained for low-grade non-invasive papillary UCs. One possible explanation for this is that low-grade non-invasive papillary tumors are frequently diploid and have relatively few chromosomal aberrations when compared to invasive UC and UC in situ. However, it is well established that deletions of part or all of chromosome 9 and of the 9p21 locus are a frequent occurrence in low-grade non-invasive papillary UC.36 To determine whether the finding of homozygous deletion of the 9p21 band might further improve the sensitivity of the assay by detecting low-grade non-invasive papillary UCs without polysomy, we analyzed the FISH false negative non-invasive papillary UC cases for abnormality for homozygous 9p21 deletion. We found that in one case, a high proportion (10 or more cells) of the selected target cells showed homozygous loss of 9p21 locus. If homozygous loss of 9p21 region in >50% of the selected target cells (when no other chromosomal abnormalities were present) as an additional criteria for cancer positivity increased the overall sensitivity of the assay from 84.2% to 86.8%, but did not change its specificity (91.8%). A comparison of FISH using this probe set (ie, the probe set containing the CEP3, CEP7, CEP17, and LSI 9p21 probes) to cytology in this patient cohort demonstrated that FISH was more sensitive than cytology for the detection of pTis (100 vs. 78%, P = 0.046), pT1-pT4 (95 vs. 60%, P = 0.025), and grade 3 (97 vs. 71%, P = 0.003) tumors. Additionally, FISH showed a trend toward a statistically significant increase in the sensitivity for the detection of pTa (65 vs. 47%, P = 0.058) and grade 2 (76 vs. 54%, P = 0.059) tumors. There was no significant difference in the sensitivity of FISH and cytology for the detection of grade 1 tumors.
Discussion

In this study we describe the development of a multitarget, multicolor FISH assay for the detection of UC. The first goal of the study was to determine an optimal set of probes for the detection of UC in urine specimens, i.e., the minimum number of probes that provides high sensitivity for UC. This optimal FISH probe set was determined by testing various probes for UC detection in urine from patients with UC and selecting those that were either the most sensitive individually or that complemented other probes to enhance the overall sensitivity of the test. The CEP7 probe was the most sensitive of the eight probes tested, and the CEP17, CEP3, and LSI 9p21 probes were the probes that best complemented the CEP7 probe to enhance the overall sensitivity of the assay. These probes were combined into a single probe set for additional studies.

Our findings clearly show that the use of more than one probe improves the sensitivity of UC detection. With the CEP7 probe alone, the sensitivity of the assay for UC would have been only 76% (16/21 patients). However, the addition of CEP3, CEP17, and the LSI 9p21 probes to the set increased the sensitivity to 95% (20/21 patients). These findings are consistent with those of Sauter et al, who have shown that the fraction of paraffin-embedded UCs demonstrating chromosomal abnormalities with FISH increases when the number of probes used is increased from one to four.37 They did not find any further increase in the fraction of tumors detected when 4 to 6 probes were applied. Although the overall sensitivity of the LSI 9p21 probe was low (29%; 6/21 patients), it enhanced the sensitivity of the assay by detecting tumors that were not detected with the CEP probes. The CEP probes and LSI 9p21 probe are complementary because the CEP probes detect hyperdiploidy, a common feature of carcinoma in situ and invasive UC, but not non-invasive papillary UC, whereas the LSI 9p21 probe detects deletions of the 9p21 band, a common feature of non-invasive papillary UC.15–21

The CEP9 probe was, surprisingly, found to be one of the least sensitive probes (Table 3). This may seem surprising since loss of all or part of chromosome 9 has been reported as the most frequent chromosomal abnormality in UC. An explanation for this paradox may be that if chromosome 9 loss occurs before tetraploidization (a common event in solid tumors), then the copy number of chromosome 9 would go from monosomy to disomy.22,23,38 Thus, the copy number of chromosome 9 would appear normal despite the fact that the cell is hyperdiploid.

Our experience with FISH has led us to realize that the finding of polysomy (i.e., gains of two or more different chromosomes) in a cell is a more specific indicator than the gain of a single chromosome. For example, our calculations show that the specificity of our assay using the criteria 5 cells with polysomy (gains of multiple chromosomes) was 91.8% (45/49 patients). If we use as a criterion for cancer positivity 5 cells with gains of a single chromosome (for example, CEP3), then the specificity of the assay drops to 81.6% (40/49 patients). This fact can be easily explained statistically, because it is less likely that the artifacts (such as a split signal) occur simultaneously on two independent chromosomes in the same cell.

We also found that a scanning method was more sensitive and faster than counting for the analysis of FISH slides. The higher sensitivity of the scanning method is probably due to the fact that it allows the observer to examine a much larger population of cells (up to thousands) for evidence of malignancy. This is not possible with counting, due to the large amount of time required to count cells. Counting may miss rare abnormal cells if the percentage of abnormal cells is low. We found this to be especially true of cases that had FISH abnormal cells hidden among inflammatory or normal epithelial cells.

The scanning technique can generally be performed in 1 to 10 minutes by an experienced observer. Specimens with high percentages of FISH abnormal cells can be categorized as positive for malignancy in approximately 1 minute; specimens with relatively few FISH abnormal cells may require up to 10 minutes to evaluate. Counting 100 cells, on the other hand, generally takes 30 to 60 minutes per case.

A receiver operator characteristic curve (Figure 3) revealed that a cutoff of 5 or more cells with polysomy as the criteria for evidence of UC gave a sensitivity and specificity of 84.2% and 91.8%, respectively. A slightly higher sensitivity (89.5%) but lower specificity (87.8%) were obtained with a cutoff of 4 or more cells. To maintain a high specificity, we recommend that the cutoff of 5 or more cells with gains of 2 or more different chromosomes be used to classify cases as malignant.

In summary, this study reveals that a FISH probe set containing probes to the centromeres of chromosomes 3, 7, and 17 and to the 9p21 band has high sensitivity and specificity for the detection of UC. Our studies suggest that the best way to assess slides is to scan the slide for cells with morphologically abnormal features and then determine whether the cell has chromosomal aberrations suggestive of malignancy by viewing the FISH signals. Our studies also suggest that the sensitivity of this FISH assay for UC is superior to urine cytology and yet maintains the high specificity of cytology. It is possible that regular surveillance of UC patients for tumor recurrence with FISH could reduce UC mortality, because false negative urine cytology results place superficial UC patients at risk of undetected progression to less curable muscle-invasive UC.

References


