Toward the Validation of Aneusomy Detection by Fluorescence in Situ Hybridization in Bladder Cancer: Comparative Analysis with Cytology, Cytogenetics, and Clinical Features Predicts Recurrence and Defines Clinical Testing Limitations

Feiyu F. Zhang, Daniel A. Arber, Timothy G. Wilson, Mark H. Kawachi, and Marilyn L. Slovak

ABSTRACT

Fluorescence in situ hybridization (FISH) is regarded as a potential new tool for the clinical management of bladder cancer that works by detecting cytogenetic aberrations in noncycling, exfoliated cells from bladder irrigations. However, clinical validation steps must be addressed to define the true predictive potential in a clinical setting. Toward the validation of FISH with the use of bladder washings and prior to incorporation into a large, prospective clinical trial, a pilot study was designed to determine its clinical potential, define testing limitations, optimize a panel of probes specific for bladder cancer detection, and outline protocol/data collection parameters. Correlations with standard cytogenetics and clinicopathological features of bladder cancer were investigated. Exfoliated cells obtained from benign bladder washings served as normal controls. The results of this pilot study suggest the following: (a) FISH and cytology are complementary testing procedures; however, the FISH data provided valuable ploidy and specific genotypic information for recurrent tumors in “suspicious” cases; (b) chromosomal aberrations defined by FISH are associated with tumor grade and stage (i.e., simple numerical aberrations were associated with low-grade tumors, and high-grade and invasive tumors exhibited multiple, nonrandom chromosomal aberrations and vast intratumor heterogeneity); (c) somatic pairing or homologous centromeric association can give a false-positive result and appears to be linked to prior therapy; (d) dual hybridization with reference gene-specific probes must be used to control for somatic pairing; and (e) focal, deep muscle invasive lesions, with no surface exposure, may yield false-negative results. The data suggest that FISH analysis, with the use of cells isolated from bladder washings, is a powerful technique holding promise for early cancer detection, monitoring treatment outcome, and predicting recurrence of disease.

INTRODUCTION

Approximately 50,000 new cases of bladder cancer are diagnosed annually in the United States, making it the fifth most common cancer in Western society (1). Ninety percent of bladder cancer cases are categorized as TCC (2) whereas the remaining 10% are classified as either squamous cell carcinoma or adenocarcinoma (3). Three subgroups of TCC have been defined by histopathology: papillary (invasive and noninvasive), nonpapillary, and Tis, each with dramatically differing clinical histories and outcomes (2-4). Histopathological classification reflects tumor grade and stage; however, it fails to reliably predict pathobiological characteristics, such as drug resistance, recurrence, and invasive or metastatic potential. A major problem in the management of superficial bladder cancer is accurate identification of those TCC cases with the highest risk of relapse and progression.

Genetic aberrations in cancer have become a primary focus to study the pathogenesis of urothelial tumors (2, 5). In bladder cancer, assessment of chromosome content by FCM or SC has been correlated with several clinical parameters, including diagnosis, histopathology, biological behavior, and prognosis. Tumor progression and recurrence appear to correlate with aneuploidy and a high proliferative rate. However, ascertainment of ploidy by FCM has limited value in low-grade tumors, because small variations in DNA content resulting from loss or gain of partial or one to two chromosomes cannot be detected. Additionally, FCM provides no data regarding specific genetic events that may occur during tumor development or progression, and most invasive tumors are aneuploid (3). Sensitivity of DNA FCM for grade 1–3 tumors ranges from 12 to 89%, with the widest range for grade 1 tumors (6, 7). Alternatively, SC provides an overview of specific chromosomal aberrations and

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2 To whom requests for reprints should be addressed, at City of Hope National Medical Center, Department of Cytogenetics, Northwest Building, Room 2255, 1500 East Duarte, Duarte, CA 91010. Phone: (626) 359-8111, ext. 2313; Fax: (626) 301-8877; E-mail: mslovak@smtplink.coh.org.

3 The abbreviations used are: TCC, transitional cell carcinoma; Tis, transitional carcinoma in situ; FCM, flow cytometry; SC, standard cytogenetics; FISH, fluorescence in situ hybridization; FISH-BW, FISH on bladder washings; FISH-PE, FISH on paraffin-embedded tissue section; Ta, papillary noninvasive tumor.
ploidy levels; however, analysis is limited by sample availability, mitotic yield of fresh tumor material, and in vitro cell processing complications (8, 9). Accordingly, genetic tests that will enhance the diagnostic accuracy of bladder cancer detection and surveillance and provide valuable prognostic information to the practicing urologist are warranted.

FISH is a rapid, powerful and sensitive technique for studying molecular cytogenetic abnormalities in malignant cells independent of cell cycling status. Furthermore, FISH permits direct correlation of selected karyotypic anomalies with cell morphology. Recent reports indicate that specific numerical alterations of chromosomes 1, 3, 5, 7, 8, 9, 10, 11, 17, and Y in bladder cancer detected by SC and FISH may be useful to screen urine or bladder washings for early tumor detection or recurrence (8, 10–21); however, clinical validation steps must be addressed to define the true predictive potential of aneusomy screening in a clinical setting. The objectives of this clinical validation study were (a) to define a panel of six chromosome-specific probes and validate its usefulness as a screen to detect aneusomy in exfoliated cells from bladder washings (FISH-BW), (b) to investigate the ability of FISH-BW to reflect true nonrandom numerical chromosomal aberrations in bladder cancer, and (c) to correlate the FISH-BW data with cytology, clinical cystoscopy findings, and histopathology.

MATERIALS AND METHODS

Samples. This study was a 1-year Institutional Review Board-approved single-institution pilot study for the Southwest Oncology Group Genitourinary Tumor Biology Program. SC were performed on 11 fresh tumors and 1 bladder washing; FISH analyses were performed on 23 paraffin-embedded tissues (18 tumors and 5 normal bladder tissue controls) and 37 bladder washings or urine. Tissue availability was the only criterion for analysis; no sample was excluded from the study on the basis of clinical profile. Clinicopathological and cytogenetic features of the cases successfully analyzed are listed in Tables 1–3. Table 1 compares SC to FISH-PE from 11 fresh bladder tumor samples, obtained at time of cystectomy or transurethral resection, and 1 bladder washing (case B21). Concurrent paraffin blocks containing the same portion of the tumor used for SC were studied by FISH-PE.

Eight tumors were collected for FISH-BW and FISH-PE comparisons (Table 2). In five cases, tumor tissue was collected on the same day as was the bladder washing specimens. Tumor tissue corresponding to cases B12 and B14 was collected and embedded 9 and 8 months, respectively, before collection of the bladder washing; tumor tissue corresponding to case B20 was collected 6 months after bladder irrigation.

For the FISH-BW comparison study, 35 bladder washings (150–200 ml) and two urine samples were obtained at the time of transurethral resections or at follow-up cystoscopy. FISH analysis was determined successful when a 200-cell count for each of the six probes was possible. Concurrent cytology for the 25 successful samples are listed in Table 3: benign disease (n = 4), reactive cytology (n = 7), suspicious for bladder carcinoma (n = 7), and bladder carcinoma (n = 7).

SC. Twelve bladder tumors, including one highly mitotic bladder washing (case B21), were cultured and harvested according to standard technique. Giemsa-trypsin chromosome banding (GTG banding) was used to identify nonrandom chromosomal aberrations. International System for Human Cytogenetic Nomenclature 1995 guidelines were followed for clonal definition and description of individual structural and numerical karyotypic anomalies.

FISH-PE. Four-μm sections were pretreated and hybridized with chromosome-specific enumeration probes for chromosomes 1 (α satellite), 7 (α satellite), 8 (α satellite), 9 (β satellite), 17 (α satellite), and Y (cocktail; Oncor, Inc.) according to the manufacturer’s instructions, with slight modifications. Tissue sections were pretreated with 30% sodium bisulfite in 2× SSC (1× SSC = 0.15 m NaCl and 0.015 m Na Citrate, pH 7.0) at 45°C for 15 min and digested in proteinase K (0.25 mg/ml in 2× SSC; Oncor, Inc.) at 45°C for 40–90 min.

FISH-BW. Exfoliated cells from bladder washings or urine samples were collected by centrifugation (400 × g for 8 min), washed with 1× PBS and exposed to 0.075 m KCl hypotonic solution at 37°C for 20 min. These cells were fixed with Carnoy’s fixative before being dropped onto silanized slides. The panel of FISH probes described above for FISH-PE was used. Hybridization and signal detection were carried out according to standard technique (Oncor, Inc.). Two probes were applied to two adjacent areas (22 × 22 mm in size) on one slide.

To rule out the possibility of somatic pairing, slides from three cases (B9, B10, and B11) previously hybridized with enumeration probes were rehybridized with either the ABL (9q34) or the HER2/neu (17q11.2–12) DNA probe (Oncor, Inc.). Coverslips were removed prior to destaining in 1× phosphate-buffered detergent for 10 min at room temperature and dehydration in ethanol (70, 80, and 95%). The initial enumeration probes were washed off, and the target DNA was redenatured by incubation at 72°C in 70% formamide/2× SSC for 1–1.5 min. Hybridization and posthybridization washes were carried out as described above.

Scoring Criteria for FISH and Statistics. FISH slides were scored using a Nikon microscope equipped with FITC/Texas Red and FITC filters. Scoring criteria for FISH-BW were defined to ensure that the data obtained from the two independent scorers were comparable: (a) 200 nuclei were scored per probe per case; (b) only intact, nonoverlapping nuclei were scored; (c) split adjacent signals less than the diameter of a signal apart were counted as one signal; (d) nuclei without hybridization signals and inflammatory cells and squamous cells with intact cell membrane and cytoplasm were recorded but not counted; and (e) nuclei with obvious cross-hybridization and/or high background were not scored. For chromosome Y signals, cells without signal were scored as chromosome loss. The following scoring criteria were used for FISH-PE: (a) a minimum of 200 nuclei from at least four different pathologically confirmed tumor areas; (b) only nonoverlapping nuclei with a size no smaller than one-third of average size and good propidium iodide staining were scored; (c) split signals were counted as a single signal; and (d) nuclei without probe signal were scored to evaluate the effects of truncation due to sectioning.

The enumeration probes for the autosomes tested were chromosome-specific repetitive DNA probes for human satellite DNAs located near the centromere. Leukemia and bladder tissue...
Table 1  A comparison of numerical chromosomal abnormalities of bladder tumors detected by FISH-PE and SC for chromosomes 1, 7, 8, 9, 17, and Y

Chromosomal abnormalities listed in Tables 1–3 represent the subpopulation containing the highest number of clonal chromosomal gains, assuming a clone equaled or exceeded 5.0% of the total cells scored in bladder washings and 4.0% in paraffin-embedded sections.

<table>
<thead>
<tr>
<th>Case</th>
<th>Grade/Stage</th>
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<th>chr 7</th>
<th>chr 8</th>
<th>chr 9</th>
<th>chr 17</th>
<th>chr Y</th>
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<th>chr 7</th>
<th>chr 8</th>
<th>chr 9</th>
<th>chr 17</th>
<th>chr Y</th>
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<tr>
<td>A1</td>
<td>II</td>
<td>Ta</td>
<td>N</td>
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<td>Ta</td>
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<td>Ta</td>
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<td>N</td>
<td>N</td>
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<td>N</td>
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<td>T3</td>
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<td>+</td>
<td>+</td>
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<td>N</td>
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<td>+</td>
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<td>+</td>
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<td>T3</td>
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<td>+,+, +</td>
<td>+,+, +</td>
<td>+,+, +</td>
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<td>+,+, +</td>
<td>+,+, +</td>
<td>+,+, +</td>
<td>+,+, +</td>
</tr>
</tbody>
</table>

a chr, chromosomal; N, normal; der, derivative chromosome; del, deleted chromosome; +, chromosome gain; the number of + indicates the chromosome gain in comparison with a normal diploid complement; −, chromosome loss, the number of − indicates chromosomal loss in relation to normal diploid; N, normal diploid number; /, female.

b Faint second signal observed due to partial loss of classical satellite DNA (structural aberration) in a single chromosome 9.

c Tetraploidization was found in one small locus, whereas 1 of 15 cells was near tetraploid by SC.

d Disomy Y was observed in the mainline.

e SC revealed 3 N in 15 of 20 cells, and 6 N, idemx2 in 5 of 20 cells.

f Many unidentifiable chromosome markers.

g Diploid cells were detected as the main population by both FISH and SC; 4N cells were also observed by FISH-PE besides 3 N (truncated 4 N cells).

Table 1 from our laboratory confirmed an earlier observation, reporting no significant differences among repetitive DNA autosome probes to detect chromosomal gains and losses (16). Thus, a common cutoff value for FISH-BW and FISH-PE was used to determine autosomal chromosome loss and gain.

Cutoff values for FISH-BW were determined using four cytologically benign samples. The percentage of nuclei with only one autosomal signal was 43 ± 1.8% (mean ± SD; P = 0.95; n = 20); the percentage of nuclei with more than two signals was 26 ± 1.94% (mean ± SD; P = 0.95; n = 20). To distinguish monosomy and polysomy from normal background, conservative cutoff values, as reported by others, were set at 3 × SD above the mean percentages from samples of benign cells with one and more than two signals, respectively (22–24). Cutoff values for monosomy and polysomy in FISH-BW were set at 10% and 9%, respectively. Because only one benign bladder washing specimen was collected from a male, the 20% cutoff for chromosome Y loss, as described by Sauter et al. (13), was used; a value of greater than 10% implied gain of chromosome Y.

Urothelium from five normal bladder samples obtained at autopsy, with no history of bladder cancer, served as controls to define cutoff values for FISH-PE. The percentage of nuclei with no or only one autosomal signal was 44.9 ± 3.3% (mean ± SD; P = 0.95; n = 25); the percentage of nuclei with more than two signals was 1.9 ± 0.9% (mean ± SD; P = 0.95; n = 25). A higher percentage of cells with no or only one autosomal signal is due to the truncation effect of paraffin-embedded tissue sectioning. A similar percentage of cells with fewer than two autosomal signals was reported previously for normal tissue (25). Applying the same criteria used for FISH-BW, cutoff values for monosomy and polysomy in FISH-PE were 55% and 5%, respectively. For chromosome Y, the percentage of nuclei with no signal was 17.2 ± 2.0% (mean ± SD; P = 0.95, n = 5); the percentage of nuclei with more than one Y signal was 2.0 ± 0.6% (mean ± SD; P = 0.95, n = 5). Thus, cutoff values of 24% and 4%, respectively, were set for gain and loss of chromosome Y.

Intratumor genetic heterogeneity is frequently observed in bladder tumors with higher ploidy levels. Chromosomal abnormalities listed in Tables 1–3 represent the subpopulation containing the highest number of clonal chromosomal gains, assuming a clone equaled or exceeded 5.0% of the total cells scored in bladder washings and 4.0% in paraffin-embedded sections. These values corresponded to clonal findings defined by SC.

RESULTS

Correlation of SC and FISH-PE. Table 1 summarizes the histopathology and numerical abnormalities identified by SC and FISH-PE for the 12 bladder cancer cases successfully analyzed by both methods. Ten cases (83.3%) were concordant. Abnormalities of chromosome 9 [monosomy 9 and del(9q)] were observed as the sole anomalies in low-grade, noninvasive TCCs; marked aneuploidy was common in the high-grade tu-

Unpublished data.
mors. In eight of nine cases, chromosome gains were detected by FISH-PE despite nuclear truncation due to sectioning. Comparable SC and FISH-PE results for a grade III T2 bladder cancer (case A7) are shown in Figs. 1 and 2A.

Two discrepant cases were identified. In case A4, only two hybridization signals for all chromosomes tested were observed by FISH-PE; however, trisomy 7 was observed by SC in 3 of 20 metaphases (15%). FISH performed on the residual SC suspension using the chromosome 7 probe indicated a 3% trisomy 7 population, a finding within normal background limits. In case A9, near-diploid cells with clonal aberrations of chromosomes 7, 8, and 9 were detected by SC; FISH-PE detected both a near-diploid and a near-tetraploid clone with similar chromosomal aberrations.

**Correlation of FISH-PE and FISH-BW.** Table 2 compares numerical abnormalities identified by FISH for eight bladder washing samples with their corresponding paraffin-embedded tissue. All samples were concordant; however, a limitation of the procedure was determined. Histopathology revealed two lesions in tumor B20, a superficial lesion (Tis) and a nonexposed invasive lesion (T2). No chromosomal aberrations were detected by either FISH-BW or FISH-PE in the Tis lesion; however, FISH-PE detected multiple numerical aberrations in the deep focal invasive T2 lesion (Fig. 3, A-C). These data indicate that false-negative results may occur when lesions are not exposed to the bladder surface. In case B24, a near-octaploid (8N) population with underrepresentation of chromosomes 9, 17, and Y was identified by FISH-BW (Fig. 2, D and E; Fig. 3F). Both FISH-PE and FISH-BW revealed a complementary aneuploid histogram in B24; however, FISH-PE was able to detect ploidy differences between the Tis and Ta lesions.

Minor variances in detection between the two FISH methods were observed. Chromosomal gains were detected by both FISH-BW and FISH-PE in case B21; however, due to truncation of nuclei by tissue sectioning, FISH-PE consistently underestimated subpopulations with chromosomal gains. In case B23, chromosome 9 loss was detected by both methods, but FISH-PE detected a minor polyploid subpopulation (10–13.5%) that was within background limits by FISH-BW. Furthermore, monosomy 9 was equivocal or suspected by FISH-BW in B18 but easily detected by FISH-PE.

**Correlation between FISH-BW and Cytology.** Twenty-five (68%) of the 37 specimens (35 bladder washings and 2 urine samples) were deemed successful, because a 200-cell analysis per probe study requirement was met. The main reason for an unsuccessful study was an insufficient number of cells for a complete analysis (n = 10); two technical failures were also noted. Voided urine samples contained too few cells for successful FISH analysis and were prone to contamination by squamous and inflammatory cells. Table 3 summarizes cytology diagnoses, cystoscopic findings, and numerical abnormalities detected by FISH-BW for the 25 samples.

Six bladder washings were diagnostic for either TCC (n = 5) or squamous cell carcinoma (n = 1). Numerical abnormalities were detected in all six samples. In two tumor cases (B22 and B23), monosomy 9 was the sole numerical anomaly detected by FISH-BW and FISH-PE (Fig. 2, B and C; Fig. 3, D and E).

In three TCCs and the sole squamous cell carcinoma case,
<table>
<thead>
<tr>
<th>Case</th>
<th>Cytology</th>
<th>Clinical findings (cystoscopy)</th>
<th>Histopathology grade/stage</th>
<th>Numerical chromosomal abnormalities by FISH-BW</th>
</tr>
</thead>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>chr 1</td>
</tr>
<tr>
<td>B1</td>
<td>Benign</td>
<td>Negative</td>
<td>NA</td>
<td>N</td>
</tr>
<tr>
<td>B2</td>
<td>Benign</td>
<td>Negative</td>
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<td>N</td>
</tr>
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<td>Benign</td>
<td>Negative</td>
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<td>N</td>
</tr>
<tr>
<td>B4</td>
<td>Benign</td>
<td>Negative</td>
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<td>N</td>
</tr>
<tr>
<td>B5</td>
<td>Reactive</td>
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<td>N</td>
</tr>
<tr>
<td>B6</td>
<td>Reactive</td>
<td>Negative</td>
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<td>N</td>
</tr>
<tr>
<td>B7</td>
<td>Reactive</td>
<td>Negative</td>
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<td>N</td>
</tr>
<tr>
<td>B8</td>
<td>Reactive</td>
<td>Negative</td>
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<td>N</td>
</tr>
<tr>
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<td>Reactive</td>
<td>Negative</td>
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<td>N</td>
</tr>
<tr>
<td>B10</td>
<td>Reactive</td>
<td>Negative</td>
<td>NA</td>
<td>N</td>
</tr>
<tr>
<td>B11</td>
<td>Reactive</td>
<td>Cancer</td>
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<td>N</td>
</tr>
<tr>
<td>B12</td>
<td>Suspicious</td>
<td>Negative</td>
<td>II, Ta</td>
<td>+,+</td>
</tr>
<tr>
<td>B13</td>
<td>Suspicious</td>
<td>Negative</td>
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<td>N</td>
</tr>
<tr>
<td>B14</td>
<td>Suspicious</td>
<td>No tumor (atypia, erythema)</td>
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<td>+,+</td>
</tr>
<tr>
<td>B15</td>
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<td>Negative</td>
<td>II, Ta</td>
<td>+,+</td>
</tr>
<tr>
<td>B16</td>
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<td>Negative</td>
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<td>N</td>
</tr>
<tr>
<td>B17</td>
<td>Suspicious</td>
<td>Possible recurrent TCC</td>
<td>Negative</td>
<td>N</td>
</tr>
<tr>
<td>B18</td>
<td>Suspicious</td>
<td>Ta</td>
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<td>TCC</td>
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<td>Cancer</td>
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<tr>
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<td>TCC</td>
<td>Cancer</td>
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<td>Ta</td>
<td>II/III, Ta/Tis</td>
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<tr>
<td>B25</td>
<td>SCC</td>
<td>Metastatic bladder cancer</td>
<td>Poorly differentiated SCC</td>
<td>+,+</td>
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* chr. chromosome; NA, not available; N., normal; ND, not done; +, gain, the number of +s indicates increased copy number over the normal diploid content; -, loss, the number of - indicates chromosome loss based on normal diploid; (%) following - indicates the size of the abnormal population; f., female; SCC, squamous cell carcinoma.

* Normal ABL gene content by FISH-BW.

* Normal HER2/neu content by FISH-BW.

* Normal Tis lesion; aneuploid infiltrated lesion not exposed to bladder surface (by FISH-PE).
chromosomes 1, 7, 8, and 17 were overrepresented. Chromosome 9 was underrepresented in case B24, as illustrated by four copies of chromosome 9 in association with eight copies of chromosomes 1, 7, and 8 (Table 3; Fig. 2, D and E; Fig. 3F), suggesting karyotypic evolution of the monosomy 9 clone by polyploidy associated with tumor progression. Loss of chromosome Y was detected in one TCC (B24) and in the squamous cell carcinoma case (B25), whereas gain of chromosome Y was detected in one invasive TCC (B21).

Seven cases each were diagnosed cytologically as “reactive” or “suspicious for tumor.” FISH-BW studies revealed normal disomy for all chromosomes tested in two cases (one reactive and one suspicious case). In four (B12, B14, B15, and B18) “suspicious” cases, numerical abnormalities were detected, supporting a diagnosis of tumor recurrence. All four cases were characterized by losses of chromosome 9.

The remaining eight reactive/suspicious cases, identified by cytology, were characterized by a single signal for either
chromosome 9 or chromosome 17 in 10–33% of cells scored, exceeding the cutoff value for chromosome loss (Table 3). Three cases involved chromosome 9 only, two cases involved chromosome 17 only, and three cases involved both chromosomes 9 and 17. To investigate the possibility of somatic pairing, FISH was repeated on samples B9, B10, and B11 using a probe for either ABL (9q34) or HER2/neu (17q11.2-12) in combination with the respective chromosome specific DNA centromeric probe. Results of this study indicate that somatic pairing, or paired signals appearing as a single hybridization, occurred in these posttreatment samples (Fig. 2F).

FISH and cystoscopic findings were 100% concordant in the benign and obvious tumor cases (Table 3). In the cytologically reactive and suspicious cases, discrepancies were observed in B12, B14, and B17. In these cases, FISH results were consistent with the histopathological diagnoses.

**Focal Polyploidization.** Focal polyploidization was evident in two grade II Ta tumors (A2 and B14) by FISH-PE. In case A2, most of the tumor was near-diploid with monosomy 9, whereas one small focus consisted of near-tetraploid cells with only two copies of chromosome 9. By SC, one near-tetraploid cell and 14 near-diploid cells were analyzed. Similar results were observed in case B14 by FISH-PE, except the polyploid focus was more extensive.

**Follow-Up Samples.** Follow-up samples were obtained from three patients. In case B19, a bladder washing was obtained at cystoscopy prior to biopsy. FISH-BW detected chro-
Fig. 3 FISH histograms detailing the distribution of hybridization signals in bladder cancer. A–C, molecular cytogenetics in case B20 using pericentromeric probes for chromosomes 7, 9, and Y from the bladder washing (A), paraffin-embedded Tis lesion (B), and paraffin-embedded deep focal Ta lesion (C). Normal chromosome copy number was evident by FISH-BW and by FISH-PE in the Tis lesion. FISH-PE of the Ta lesion detected two copies of chromosome 9 in an otherwise tetraploid tumor, as noted by two copies of the Y chromosome and trisomy/tetrasomy for chromosome 7. Data indicate that the Ta lesion evolved from a paradiploid tumor characterized by monosomy 9, D and E, loss of chromosome 9 in case B22. Similar distribution of hybridization signals for chromosomes 7, 9, and Y are illustrated in the bladder washing (D) and the paraffin sections (E). A single signal is observed for both chromosomes Y and 9, whereas two signals are recorded for chromosome 7 in this paradiploid Ta tumor. F, distribution of FISH signals in case B24 for chromosomes 7, 9, and Y. Data indicate loss of chromosome Y, an evolved monosomy 9 tumor, and marked aneuploidy and intratumor heterogeneity with cells containing more than six copies of chromosome 7. FISH illustrations of this tumor are presented in Fig. 2, D and E.

mosome gains for all chromosomes tested (including chromosome 17), supporting the histopathological diagnosis of Tis. The patient was treated with 12 weeks of Bacillus Calmette-Guérin intravesically. At follow-up, the posttherapy bladder washing (case B7) exhibited a normal chromosomal content with equivocal loss of chromosome 17 signal consistent with somatic pairing. FISH results supported the cytodiagnosis in both samples.

Two bladder washings from a recurrent grade II, Ta bladder cancer (B12 and B15) were obtained 3 months apart. FISH-BW detected similar chromosome gains with underrepresentation of chromosome 9 in both samples, consistent with recurrence of a low-grade Ta.

A concurrent bladder washing obtained at the time of the diagnostic grade II, Ta lesion (case B22) detected loss of chromosome 9 in 89.5% of the exfoliated cells. At follow-up 8 months later (case B18), FISH-BW again identified monosomy 9 in 15.5% of cells, whereas the cytodiagnosis was “suspicious” but not definitive for recurrent disease. Cystoscopy and histopathology revealed a low-grade Ta.

DISCUSSION

FISH is regarded as a potential tool for clinical management of bladder cancer that works by detecting cytogenetic aberrations in noncycling cells using exfoliated cells from bladder irrigations (8, 10, 17–19, 21, 26–28). To validate FISH-BW as a clinical tool prior to its incorporation into a large prospective clinical trial, a pilot study was initiated to determine clinical utility, define testing limitations, optimize a probe panel specific for bladder cancer detection, and delimit protocol/data collection parameters. In this study, a comparison of the FISH-PE data to SC validated detection...
of specific chromosomal gain or loss in a particular tumor. In cases in which fresh tumor material was not available, paraffin-embedded material was used to identify specific chromosomal aneusomy in monitoring clinical course of disease. Because the SC, FISH-BW, and FISH-PE methods were concordant, FISH-BW was shown to be a reliable and sensitive molecular cytogenetics approach that can monitor the nonrandom chromosomal aneusomy frequently observed in bladder cancer.

Urine cytology is widely used to detect and monitor urothelial tumors; however, this methodology suffers from low sensitivity (56%), in part due to the morphological resemblance of low-grade papillary tumors to normal urothelium and also to therapy-related complications associated with chemotherapy and radiation (17, 29, 30). In this study, approximately 30% of the cases were diagnosed as suspicious, an inconclusive cytopathology for bladder cancer. Cystoscopy findings on these cytologically suspicious cases were either falsely negative or inconclusive. Using a panel of six probes, FISH showed complete concordance with cytology in benign and unequivocal tumor samples. Moreover, FISH detected obvious numerical aberrations (polyploidy) in 43% of cases with "suspicious" cytodiagnosis. Assuming that additional comparative studies confirm this seeming increase in sensitivity, without loss of specificity, for FISH-BW, continued exploration of developing multiplex FISH technology using urine samples may allow stratification of patients based on urothelial cell genotype. This developing technology has the potential to decrease the frequency of follow-up cystoscopy for patients with negative cytological examinations in the absence of a concomitant bladder tumor.

The establishment of appropriate scoring criteria is essential to achieve maximum sensitivity and specificity in FISH-BW. Bladder washings often contain inflammatory cells that could exceed half the exfoliated cells in some specimens. In these cases, it is critical to recognize and not score inflammatory or squamous cells to avoid a false-negative result. Inflammatory cells are distinguishable from bladder cells by morphology; thus, prior review of the corresponding cytology slides improves the accuracy of the FISH assay.

Careful selection of FISH probes is key to providing valuable prognostic information. On the basis of the nonrandom involvement of specific chromosomes and their apparent clinical significance as reported previously in SC and molecular cytogenetic studies, centromeric probes for chromosomes 1, 7, 8, 9, 17, and Y were chosen (8, 10, 13, 16, 17, 19–21, 26, 31, 32). Using this panel, additional copies of chromosomes 1 and 8 were frequently detected concomitantly with an increase in ploidy level; commonly overrepresented copy numbers may thus be reliable ploidy indicators for bladder carcinomas. Loss or gain of chromosome Y is also a frequent nonrandom numerical aberration. In this study, both over- and underrepresentation of chromosome Y were observed. In two tumors, nullisomy Y was detected, along with ploidy and numerical changes suggesting that loss of chromosome Y occurred prior to polyploidization (13). Reports indicate that loss of the Y is commonly associated with complex karyotypes and poor overall survival (32); however, loss of Y, as the sole karyotypic aberration, has been reported in low-grade, noninvasive tumors. Unlike the case in hematopoietic stem cells, loss of the Y chromosome in bladder cancer appears not to be age related (32). The frequent involvement of chromosome Y, and its association with a poor prognosis, makes it an excellent FISH probe to detect bladder cancer.

Trisomy 7, as the sole karyotypic aberration, was detected by SC in one case; however, this finding was not confirmed by FISH-PE. Trisomy 7, by itself, has been associated with tumor-infiltrating lymphocytes in both tumor and surrounding nonepithelial tissue (33–37), suggesting that simple trisomy 7 is not solely representative of the tumor. The sample submitted for SC in this case may have contained pockets of infiltrating lymphocytes with a positive selection for in vitro growth that has no counterpart in FISH-PE or was negligible after tissue sectioning. Alternatively, chromosome 7 was overrepresented (one FISH-BW and three SC studies) in four high-grade, poorly differentiated tumors. All four polyploid cases were associated with other karyotypic aberrations. Gains of chromosome 7 support the association of elevated expression of the epidermal growth factor receptor gene (located on chromosome 7) by a gene dosage effect in high-grade, high-stage tumors (5, 8, 11). Taken together, chromosome 7 appears to be an informative prognostic marker associated with aggressive, high-grade tumors and an unfavorable outcome, making it a key probe to study bladder tumors.

Loss of chromosome 9 is the most commonly described abnormality in TCC. Monosomy 9, as the sole numerical aberration, was detected by FISH in four of six diploid Ta tumors. Complete loss of one chromosome signal was observed in two cases; partial loss of chromosome 9 with a breakpoint in the probe heterochromatic region was detected in the other two cases. Faint chromosome 9 signals suggestive of a chromosome 9 structural rearrangement were also reported by Hopman et al. (16). Five of seven aneuploid Ta specimens had underrepresentation of chromosome 9. Loss of heterozygosity studies and cytogenetics have suggested that 9p12–13, 9p21–22, 9q12–34.1, and 9q22 are commonly deleted regions in bladder cancer (38–41). These data support the hypothesis that chromosome 9 harbors multiple tumor suppressor genes and loss of these genes often occurs early in the course of tumor development (38, 42).

Polyploidization is infrequent in low-grade tumors but is common in high-grade tumors (3, 6, 7). An analogous relationship is found between Ta superficial and T2–3 invasive tumors (3). A higher ploidy number found in the Tis lesion, and more advanced stage associated with invasiveness, than the Ta lesion in one patient (B24) provides direct evidence for the association of ploidy level with tumor aggressiveness. In case B14, the intratumor heterogeneity findings suggest the polyploid population possesses a growth advantage over the near-diploid tumor cells, indicating that these cells may be more susceptible to additional genetic alternations associated with tumor progression and clonal evolution of disease. Clonal evolution was evident in the tetraploid focal region, characterized by loss of copies of chromosome 9, observed against the near-diploid background in case A2. This observation is of interest, because focal polyploidization in lower-grade tumors has been implicated as a clone with metastatic potential (42). Unlike FCM, FISH is a sensitive technique with the power to identify focal polyploidization.
Prospective clinical trials should be helpful to establish the metastatic potential of near-diploid tumors with focal polypliodization.

In this study, three limitations of FISH-BW for clinical testing were determined: (a) insufficient cells for analysis; (b) the false-negative sample due to deep, nonexposed tumors; and (c) false-positive results related to somatic pairing or homologous chromosome-paired signals. The most common problem was low cell yield, a restriction reported by other investigators (19, 20). Because our data analysis required a 200-cell count per probe, 10 unsuccessful samples fell into this category, including three benign, three reactive, and four suspicious cases by cytology. All malignant bladder washings provided a sufficient number of cells for FISH. To increase the success rate, fewer postfixation washes prior to dropping the cell suspension onto dry silanized slides may be critical to salvaging exfoliated cells. Additionally, multiplex FISH, using combinational fluorochromes, may decrease the number of cells/slices necessary for successful analysis.

In case B20, the FISH-BW and FISH-PE comparison indicated FISH-BW could not detect chromosomal aberrations when focal invasive tumors are not exposed to the bladder wall, giving a false-negative result. Because most invasive bladder tumors do have lesions extending from the bladder surface to the inside of the bladder wall, false-negative results will be an uncommon, but clinically significant, problem.

Somatic pairing or homologous centromere association is a poorly understood phenomenon previously observed in normal brain, prostate, and fetal tissues, in addition to malignant tissue, presupposing a connection with cell type, differentiation state, cell cycle stage, malignant evolution or genetic composition (43–49). In this study, equivocal FISH data were obtained in a significant number of cytological reactive (86%) and “suspicous” (43%) samples using the chromosome 9 classical satellite probe (10–31%) and chromosome 17 α-satellite probe (10–33%). All other enumeration probes tested fell consistently within the normal range. Because alterations of the short arm of chromosome 17 are frequent in bladder cancer, but complete loss of chromosome 17 is not common (31, 50–52), the high frequency of monosomy 17 (4 of 13 cases) in “reactive” and “suspicous” bladder washings was suspect. Somatic pairing was implicated in a FISH-BW study using repetitive DNA probes for chromosomes 9 and 15 by Wheelless et al. (21) and reported in a prostate tissue study using the same DNA repetitive probes for chromosomes 9 and 17 (43, 44). To resolve this issue, FISH studies using ABL (9q34) and HER2/neu (17q11.2–12) reference probes on samples B9, B10, and B11 reflected somatic pairing of chromosomes 9 and 17 in these urothelial cells. The equivocal cases were all posttreatment samples without evidence of disease by either histopathology or cytoscopy. These data suggest that somatic pairing may be a cellular response to cytotoxic therapy that mimics chromosomal loss and is limited to particular DNA repetitive pericentromeric probes. Without the use of control or reference probes for the chromosome 9 classical satellite DNA probe and the α-satellite DNA probe for chromosome 17, an incorrect false-positive result or equivocal clinical interpretation would have resulted. Alternatively, a translocation event, followed by subsequent loss of the centromeric portion of the chromosome, is a possibility that cannot be ruled out by FISH analysis. Concurrent SC studies would be necessary to detect and corroborate any structural rearrangements of these chromosomes.

On the basis of these encouraging findings and caveats, FISH-BW will be incorporated into a large, prospective (Phase III) clinical trial to evaluate its diagnostic efficacy and accuracy in detecting and predicting tumor recurrence in bladder cancer. Increasing the sensitivity of FISH-BW or combining urinary cytology with FISH protocols, especially in grade I TCC tumors, in which DNA flow studies are widely variable and insufficient cell numbers often preclude analysis, has the potential to improve detection of tumor recurrence in these low-grade tumors. Furthermore, development of gene-specific probes associated with bladder cancer pathogenesis in combination with multiplex FISH may result in improved surveillance for bladder cancer using voided urine as a non-invasive specimen source. The exploration for noninvasive, cost-effective approaches to assess urothelium for an underlying malignancy, or to predict recurrence and progression of disease, while excluding morphological changes induced by inflammation or therapy, should include a FISH-based genotypic profile that may stratify patients into optimal treatment regimens based on the specific genetic aberrations identified in the tumor.

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Clinical Cancer Research

Toward the validation of aneusomy detection by fluorescence in situ hybridization in bladder cancer: comparative analysis with cytology, cytogenetics, and clinical features predicts recurrence and defines clinical testing limitations.

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