Utility of a multiprobe fluorescence in situ hybridization assay in the detection of superficial urothelial bladder cancer

Mercedes Marín-Aguilera[a,b], Lourdes Mengual[a,b], María José Ribal[a], Moisès Burset[a,b], Yolanda Arce[b], Elisabet Ars[b], Artur Oliver[b], Humberto Villavicencio[b], Ferran Algaba[b], Antonio Alcaraz[a,*]

[a]Department of Urology, Hospital Clínic, Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Universitat de Barcelona, Villarroel 170, 08036 Barcelona, Spain
[b]Laboratory of Molecular Biology, Departments of Pathology and Urology, Fundació Puigvert, Universitat Autònoma de Barcelona, Barcelona, Spain

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Abstract

We evaluated the performance of a multiprobe FISH (fluorescence in situ hybridization) assay for noninvasive detection of superficial urothelial carcinoma (UC) in the bladder, in comparison to urinary cytology. Voided urine samples from 74 patients with superficial UC were analyzed by both techniques. Urine samples from 19 patients with muscle-invasive tumors and from 19 healthy control subjects were also studied. For FISH analysis, labeled probes for chromosomes 3, 7, 9, and 17 were used to assess chromosomal abnormalities indicative of malignancy. We found a significant difference between the overall sensitivity of FISH and cytology in superficial UC detection (70.3 versus 35.1%, respectively; $P < 0.0001$). This significant difference was maintained when superficial UCs were broken down into low grade (52.8 versus 13.9%, respectively; $P < 0.0005$) and high grade (86.8 versus 55.3%, respectively; $P < 0.0015$) tumors. Overall specificity was 100% for cytology and 94.7% for FISH (difference not significant). Of patients with suspicious cytology, 69% were positive by FISH. Together, these findings suggest that FISH assay for chromosomes 3, 7, 9, and 17 has a higher sensitivity than cytology and a similar specificity in the detection of superficial UC—which could be useful for reducing some cystoscopies in the accurate follow-up usually performed in these patients. © 2007 Elsevier Inc. All rights reserved.

1. Introduction

At initial diagnosis, ~90% of bladder urothelial carcinomas (UCs) are superficial (pTa, pT1, and pTis). Unfortunately, the rate of recurrence ranges from 60–85% in the 2 years following tumor resection, and progression to muscle-invasive disease accounts for 15–23% of recurred cases [1]. In consequence, long-term and close surveillance of patients with superficial tumors is of primary importance in order to manage tumor recurrences and to prevent the invasive form of the disease.

Cystoscopy and cytology are the standard methods to detect and monitor bladder tumors. Cystoscopy is an invasive technique that has a high sensitivity, except in cases of flat malignancies such as pTis. Cytology has the advantage of being noninvasive with a high specificity, but it lacks

sensitivity and produces high rates of suspicious cases, especially for low-stage and low-grade tumors [2]. Therefore, a reliable and noninvasive method for detecting UC is desirable.

Numerous morphology-based, biochemical, and molecular methods are available for diagnosing UC in urine samples [3–7]. Most of these methods, however, are not sensitive or specific enough [8–10], which has hindered them from being accepted as routine tools to displace or, at least, improve cytology and cystoscopy for the detection of bladder cancer. Fluorescence in situ hybridization (FISH) is used to directly detect cytogenetic abnormalities in malignant cells. Previous studies have revealed a number of frequent genetic changes in UC, such as increased copy numbers of chromosomes 1, 3, 7, 9, 11, and 17 [11–14] and deletions or total loss of chromosome 9 [15,16]. The study of some of these chromosomal abnormalities by FISH methodology in voided urine specimens could allow a noninvasive detection of bladder cancer.

* Corresponding author. Tel.: +34-93-2275545; fax: +34-93-2275545.
E-mail address: aalcaraz@clinic.ub.es (A. Alcaraz).

In 1998, Sokolova et al. [17] reported development of a FISH assay with high sensitivity and specificity for UC detection, using four labeled probes: three centromeric probes for chromosomes 3, 7, and 17 and one locus-specific probe for chromosome 9. These probes were eventually combined into a single multiprobe cocktail branded UroVysion (Abbott Molecular/Vysis, Des Plaines, IL). Although this assay has been tested by many groups [13,18–21], it is not yet widely used by urologists in daily practice, and more studies are needed to determine the usefulness of this molecular diagnosis in managing patients with superficial UC. Our objective was to determine the sensitivity and specificity of FISH as a noninvasive, multitarget assay for the detection of bladder UC, with particular emphasis on detecting superficial tumors.

2. Materials and methods

2.1. Patient population and samples

A total of 112 voided urine specimens (85 from men; 27 from women) obtained between January 2003 and February 2004 were included in this study. These included 74 samples from patients (mean age, 71 years) with superficial tumors and 19 samples from patients (mean age, 75 years) with muscle-invasive tumors. The 74 superficial tumors were classified as follows: 4 of pTis, 50 of pTa (33 low grade, LG; 17 high grade, HG), and 20 pT1 (3 LG; 17 HG). All 19 muscle-invasive tumors were pT2HG. Of the combined 93 patients, 20 had an associated pTis (2 pTaLG, 5 pTaHG, 7 pT1HG, and 6 pT2).

For controls, 19 samples from healthy subjects were also studied [22,23]. Voided urine specimens from all bladder cancer patients were collected prior to therapeutic surgery, and biopsy-proven UC was used as the gold standard for evidence of disease. The hospital ethics committee approved this study and both patients and control subjects provided their informed consent before participating in the study.

All urine specimens were divided into two aliquots to be analyzed by the two techniques, cytology and FISH. For cytological analysis, samples were processed on the same day that they were obtained. For FISH analysis, samples were processed within 24 hours of collection or were kept at 4°C in a preservative solution (2% polyethylene glycol–50% ethanol) until processed.

2.2. Cytology

Urine cytologies were performed according to Papanicolaou's staining and were evaluated by an expert pathologist (F.A.) blinded to the patient's clinical history. Cytologies were classified as positive, negative, or suspicious. Suspicious cytology was defined as samples containing cells with morphologies not clearly classifiable as tumoral cells or as normal cells. For the calculation of sensitivity and specificity, suspicious cytologies were scored as negative, because at our center they do not receive any curative therapy.

2.3. Sample preparation for FISH

For each sample, between 30 and 120 mL of voided urine was collected and centrifuged at $600 \times g$ for 10 minutes at room temperature. The cell pellet was resuspended in phosphate buffer saline and centrifuged at $600 \times g$ for 10 minutes. Sedimented cells were resuspended in 8 mL of fresh Carnoy's fixative (3:1 by volume methanol–glacial acetic acid). After subsequent washing with Carnoy's fixative, and except for cases with a small pellet, samples were stored in 1–1.5 mL of Carnoy's solution at −20°C. Approximately 30 μL of cell suspension was dropped onto a standard glass slide and allowed to dry. Slides were observed with a phase-contrast microscope, and a region with the appropriate cellularity was selected. Slides were then pretreated using a pretreatment kit (Abbott-Vysis) according to the manufacturer's instructions.

2.4. FISH

Slides were subjected to hybridization with the multitarget, multicolor FISH test UroVysion and the HYBrite system (Abbott-Vysis) according to the manufacturer's instructions. The UroVysion probe mixture consists of fluorescent labeled probes for the pericentromeric regions of chromosome 3 (CEP3, labeled in red), 7 (CEP7, labeled in green), and 17 (CEP17, labeled in aqua), and a locus-specific probe for chromosome 9 (LSI 9p21, labeled in gold). After hybridization, slides were washed three times at 45°C for 10 minutes in 50% formamide solution, once in 2× saline sodium citrate (SSC) for 10 minutes, and once more in 2× SSC–0.1% NP-40 for 5 minutes. Finally, 7 μL of 4′,6-diamidino-2-phenylindole (DAPI II; Abbott-Vysis) was added.

All samples were evaluated by two independent observers blinded to all cytology, cystoscopy, and biopsy results. In case of disagreement, both observers evaluated the sample again at the same time. Slides were assessed by scanning for cytologically atypical nuclei (large nuclear size, irregular nuclear shape, and patchy DAPI staining) and then determining the number of CEP3, CEP7, CEP17, and LSI 9p21 signals in those nuclei.

The criteria for FISH positivity used were those suggested by Halling et al. [18]. Briefly, a sample was considered FISH positive for UC if at least one of the following criteria was met: (i) identification of ⩾5 nuclei with gains in two or more different chromosomes (3, 7, or 17), (ii) identification of ⩾10 nuclei with the same polysomy in one chromosome (3, 7, or 17), or (iii) observation of homozygous deletion of 9p21 in ⩾20% of the nuclei counted. When one of the criteria was met, the counting process

was stopped. If none of the criteria for FISH positivity were met, ≥100 selected nuclei were scored.

2.5. Statistical analysis

Sensitivity of cytology and FISH was determined separately for the 36 and 38 patients with LG and HG superficial tumors, respectively, as well as for the 19 patients with muscle-invasive tumors. Specificity was calculated for the 19 healthy control subjects. The McNemar test was used to determine the statistical difference between the two techniques. A P-value < 0.05 was considered to indicate statistical significance.

3. Results

Single voided urine samples were collected from 74 patients with superficial UC. Overall sensitivity of FISH and cytology in the detection of these tumors was 70.4 versus 35.1%, respectively ($P < 0.0001$) (Table 1). Sensitivity of FISH for detecting LG and HG superficial tumors was higher than the sensitivity of cytology in these cases (13.9 versus 52.8% for LG tumors ($P = 0.0005$), and 55.3 versus 86.8% for HG tumors ($P = 0.0015$), respectively). A significant difference was also obtained for the comparison of FISH and cytology sensitivities in pTa tumors (64 versus 22%, respectively; $P < 0.0001$). In contrast, the sensitivity of FISH was not significantly greater than that of cytology for detection of pT1, pTis (not associated with another tumor), and muscle-invasive tumors. Overall sensitivity of cytology versus FISH in the 93 patients with UC was 47.3% (44 of 93) versus 76.3% (71 of 93) ($P < 0.0001$) (Table 1).

FISH yielded false negative results for 22 of the 74 patients with superficial UC (15 pTaLG, 3 pTaHG, 2 pT1LG, 1 pT1HG, and 1 pTisHG), and cytology yielded false negative results for 48 of the 74 patients (28 pTaLG, 11 pTaHG, 3 pT1LG, 4 pT1HG, and 2 pTisHG). For the 19 muscle-invasive cases, no false negatives were obtained by FISH technique and only one positive case was misclassified by cytology.

Although sensitivity of FISH was higher than that of cytology in the detection of the 24 pTis cases (both exclusive and associated pTis), the difference between the two techniques was not significant (79.2 versus 91.7%, respectively; $P = 0.416$). As for healthy volunteers without UC, the specificity of cytology was 100% (19 of 19; includes 1 suspicious result) and that of FISH was 95% (18 of 19; 1 false positive); the difference was not significant (Table 1).

The most common FISH criterion found in the positive urine samples ($n = 72$) was the gain of two or more chromosomes in ≥5 cells (66 of 72; 92%), followed by the criterion of homozygous deletion of 9p21 in ≥20% of the counted nuclei ((5 of 72; 7%). No case was positive on the basis of ≥10 cells with gains of a single chromosome, excepting only the false-positive control, which had gains of chromosome 7 in >10 nuclei.

As regards equivocal cytologies, 16 cytologies (17%) from the 93 patients were scored as suspicious: 12 pTa (8 LG; 4 HG), 3 T1 (2 LG; 1 HG), and 1 pT2HG. As expected, the proportion decreased as tumor stage and grade increased (Table 2). FISH analysis on these suspicious cytology samples showed a positive result in 11 of the 16 cases (69%). The five cases not detected by FISH were from patients with superficial tumors; four of the five were from LG tumors (three pTaLG, one pTaHG, and one pT1LG). The only sample from the healthy control subjects that had a suspicious result by cytology was negative by FISH.

4. Discussion

The standard procedure for early detection of recurrent superficial UC includes a combination of cystoscopy and urinary cytology [24]. The high rate of recurrence of these tumors, together with the frequent equivocal results obtained by urinary cytology and the limited utility of

Table 1
Comparison of urinary cytology and FISH sensitivities and specificities for bladder UC detection

Tumors	Cytology		FISH		
	n/N	%	*n/N*	%	*P*-value
Sensitivity: Superficial tumors (*N* = 74) (*n* represents detection positive)					
Grade					
Low	5/36	13.9	19/36	52.8	0.0005*
High	21/38	55.3	33/38	86.8	0.0015*
Stage					
Pta	11/50	22.0	32/50	64.0	<0.0001*
pT1	13/20	65.0	17/20	85.0	0.1336
pTis	2/4	50.0	3/4	75.0	1
Total superficial	26/74	35.1	52/74	70.3	<0.0001*
Sensitivity: Muscle-invasive tumors (*N* = 19) (*n* represents detection positive)					
High grade	18/19	94.7	19/19	100.0	1
Total tumors	44/93	47.3	71/93	76.3	<0.0001*
Specificity: Controls (*N* = 19) (*n* represents detection negative)					
Healthy	19/19	100%	18/19	94.7	0.6776

Abbreviations: FISH, fluorescence in situ hybridization; UC, urothelial carcinoma.

* Significant at $P < 0.05$.

Table 2
FISH sensitivity in suspicious cytologies

Tumors	Suspicious cytology		FISH sensitivity for suspicious cytology	
	n/N	%	*n/N*	%
Superficial (*N* = 74)				
Low grade	10/36	27.8	6/10	60
High grade	5/38	13.2	4/5	80
Total superficial	15/74	20.3	10/15	66.7
Muscle-invasive (*N* = 19)				
High grade	1/19	5	1/1	100
Total tumors (*N* = 93)	16/93	17	11/16	69

cytology for detecting low stage and grade UC [20,25], implies that regular invasive cystoscopies are still required to diagnose and monitor patients for recurrence and progression. Other, noninvasive diagnostic tools that overcome these limitations would be of great interest.

In a recent systematic review of urine markers for UC detection, van Rhijn et al. [26] presented evaluations of several urine-based tests with higher sensitivity than urinary cytology. One of these tests, approved by the U.S. Food and Drug Administration (FDA), is UroVysion, a test that has shown higher sensitivity for the detection of UC than urinary cytology [19,27]. Although this assay seems to be of clinical utility, mainly in superficial cases that tend to recur, it is not yet widely used in routine clinical practice—perhaps because urologists are not familiar with this technology and thus are not confident about its utility in UC diagnosis. Additional studies are required to further clarify the clinical utility of this test.

In the present study, the performance of urinary cytology and that of the noninvasive FISH assay UroVysion were compared to detect superficial UC in voided urine. To our knowledge, this study included the largest collection of voided urine samples from patients with histologically confirmed superficial bladder UC. Our results show that the UroVysion assay has higher sensitivity than cytology for the detection of superficial UC. In particular, the FISH technique showed a significantly higher sensitivity than cytology in the detection of pTa cases, as previously described [21,28]. In addition, when superficial cases were broken down by grade, FISH had a significantly higher sensitivity than cytology in the detection of both LG and HG cases. For muscle-invasive tumors, however, However, the sensitivity obtained by both techniques is similar, as reported previously [28]. We have also corroborated findings from previous studies reporting higher overall sensitivity of FISH than of urinary cytology [20,21,24,29,30].

It is widely known that pTis tumors, although superficial, have the greatest likelihood of progression and death if undiagnosed and untreated. We found sensitivity of FISH to be higher than that of cytology in the detection of superficial tumors (either exclusive or associated pTis), but not significantly higher. It may be that our pTis sample size ($n = 24$) was not large enough to allow us to find significant differences between the two techniques.

Despite the high sensitivity obtained by FISH in the 74 superficial tumors, 22 of them (29.7%) went undiagnosed with this technique. The lack of sensitivity of UroVysion in these cases could be explained by the high rate of diploid pTa tumors, as demonstrated by flow cytometry studies [31]. In addition, undetected LG samples could be explained by the low frequency of polyploidy found in LG tumors [32]. Another factor that may explain the lack of sensitivity of UroVysion is that this test is limited to just four chromosomes (3, 7, 9, and 17), which are among the most frequently altered in bladder cancer but are not the only ones [33]. It is remarkable that the increment in

sensitivity of UroVysion with respect to cytology for the detection of UC is obtained without altering its specificity.

The present findings support the idea that not all FISH aberrations are equally important [21]. No patient was positive due to the presence of ≥10 cells with gain of a single chromosome, although there was a false-positive among the healthy control subjects with gain of chromosome 7 in >10 nuclei. In addition, only 7% of positive cases met the criterion of homozygous deletion of 9p21 in ≥20% of counted nuclei. These samples were all from pTa tumors, which is in accord with the hypothesis that deletion of 9p usually occurs early in the genesis of bladder cancer [34]. Perhaps if we had counted ≥100 selected nuclei in each preparation, instead of only when none of the criteria for FISH positivity were met, we might have found this alteration in many more cases.

This molecular diagnostic test could be particularly useful in the presence of suspicious cytologies. In this study, 69% of suspicious cytologies were positive by UroVysion, allowing a clear and unequivocal diagnosis in these cases. These findings support previous suggestions that UroVysion can effectively eliminate ambiguous cytology results [19,21,29].

A surveillance program with a combination of cystoscopy and cytology after superficial tumor resection should take the initial stage and grade into account. Some authors even suggest avoiding cystoscopy, using only cytology in the knowledge that a delay of 6 months in the diagnosis of a superficial LG tumor does not adversely affect the patient [35]. Such a less invasive approach would be supported by a technique such as FISH, which improves the sensitivity of cytology in this subset of patients resulting in missing fewer tumors. We should also reconsider the need for systematic cystoscopies in the surveillance of patients with superficial HG tumors, given the availability of a noninvasive test such as UroVysion, with a sensitivity approaching 100% in these cases.

In conclusion, the greater sensitivity of FISH, compared with cytology, especially in superficial tumors, and its equal specificity means that the UroVysion FISH assay could be used as a noninvasive tool to monitor patients with superficial UC. Using cytology in combination with FISH could decrease the frequency at which cystoscopies are performed. Further randomized prospective studies are required to determine if UroVysion can be used as an alternative to cystoscopy during follow-up of high-risk bladder tumors.

Acknowledgments

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