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Improved Detection of Urothelial Carcinomas with Fluorescence Immunocytochemistry (uCyt+ Assay) and Urinary Cytology: Results of a French Prospective Multicenter Study

Eric Piaton, Laurent Daniel, Véronique Verriele, Isabelle Dalifard, Ute Zimmermann, Karine Renaudin, Françoise Gobet, Anne Caratero, Dominique Desvaux, Yves Pouille, and Daniel Seigneurin

INSERM U.407/Laboratoire d'Histologie, Faculté de Médecine Lyon Grange-Blanche (EP); Service d'Anatomie Pathologique et Neuropathologie, Hôpital de la Timone, Marseille (LD); Laboratoire d'Anatomie Pathologique et Laboratoire de Radio-Immuno Essai, CRLCC Paul Papin, Angers (VV, ID); Laboratoire d'Anatomie et Cytologie Pathologiques, Hôpital Ambroise Paré, Boulogne Billancourt (UZ); Service d'Anatomie Pathologique A, CHU Hôtel Dieu, Nantes (KR); Service d'Anatomie et Cytologie Pathologiques, Hôpital Charles Nicolle, Rouen (FG); Laboratoire d'Histologie-Cytologie, CHU Rangueil, Toulouse (AC); Département de Pathologie, Service d'Histologie-Embryologie, CHU Henri Mondor, Créteil (DD); Laboratoire Ferring S.A.S., Gentilly (YP); and Laboratoire de Cytologie, Département de Biologie et de Pathologie de la Cellule, Hôpital Albert Michallon (DS), Grenoble, France

SUMMARY: The aim of the study was to assess the sensitivity and specificity of fluorescence immunocytochemistry (uCyt+ assay) as combined with urinary cytology for detection of primary and recurrent urothelial carcinomas. We analyzed 694 urinary samples from 236 new symptomatic patients and 458 patients followed after transurethral resection (TUR) for bladder tumor. Lesions suspicious for cancer at cystoscopy were sampled by biopsies or TUR. Sensitivity and specificity of tests were calculated using cystoscopy and histopathology, whether or not combined as gold standards. In new symptomatic patients, sensitivity of uCyt+ was 40%, 88.2%, and 76.7%, whereas that of urinary cytology was 30%, 70.6%, and 83.3%, respectively, in G1, G2, and G3 tumors. In follow-up cases, sensitivity of uCyt+ was 61.9%, 66.7%, and 76.9%, whereas that of urinary cytology was 38.1%, 58.3%, and 64.1%, respectively, in G1, G2, and G3 tumors. The combination of uCyt+ and urinary cytology significantly increased mean sensitivity in newly diagnosed cases (86.4% versus 71.2% with urinary cytology only, $p < 0.05$), as well as in patients followed after TUR (79.3% versus 55.2%, $p < 0.001$). Specificity of uCyt+ and urinary cytology was identical in new patients (83.3%) and was 81.9% and 86.2%, respectively, in patients followed after TUR. In patients with negative cystoscopy, positive uCyt+ tests had a strong predictive value for tumor recurrence at 1 year (47.0% versus 11.9% in patients with negative assay, $p < 0.01$). We conclude that combining uCyt+ with urinary cytology improves the detection of urothelial carcinomas as well in patients with symptoms suggesting bladder cancer as in those followed after treatment. (*Lab Invest 2003, 83:845–852*).

Bladder cancer is the fifth most common cancer in the European Union, with over 85,000 newly diagnosed cases and about 30,000 deaths per year (Ferlay et al, 2000). Urothelial carcinomas represent 90% of bladder cancer cases, while the remaining 10% are epidermoid carcinomas or adenocarcinomas. The prognosis of urothelial carcinomas depends on both tumor grade and clinical stage of the disease: At the time of diagnosis, 75% of the tumors are superficial, ie, stage pTa, pT1, or pTIS tumors. Overall, 50% to 80% of urothelial carcinomas will recur, whereas 15% to 25% will progress to muscle invasion (Jordan et al, 1987).

Most patients with bladder cancer are treated by transurethral resection (TUR). Thereafter, a majority must be followed by cystoscopy, including or not urinary cytology in the monitoring for tumor recurrence. Most protocols used require cystoscopy to be undertaken every 3 months for 1 to 2 years, every 6 months for the next 2 years, and yearly for 10 years thereafter.

Whether on initial presentation or during the follow up, urinary cytology still remains the noninvasive assay of choice. However, a number of studies have demonstrated that urinary cytology has a low sensitivity in detecting bladder lesions, except in high grade tumors, which are diagnosed in about 80% of cases (Bastacky et al, 1999).

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Address reprint requests to: Dr. Eric Piaton, Laboratoire de Cytopathologie, Bâtiment 1, Hôpital Edouard Herriot, 69437 Lyon Cedex 03, France. E-mail: eric.piaton@chu-lyon.fr

A variety of urinary assays have been developed in an attempt to better diagnose and monitor patients with urothelial carcinomas in a fashion less invasive and more sensitive than cystoscopy or urinary cytology. However, no test has gained wide acceptance in routine urology practice. This is partly due to a lack of assurances of reproducibility, standardization, and validation in prospective studies.

uCyt+ (DiagnoCure, Sainte-Foy, Quebec, Canada) is a fluorescence immunocytochemical assay that uses a mixture of three monoclonal antibodies. Antibody 19A211, labeled with Texas Red, detects a high molecular weight form of carcinoembryonic antigen; antibodies M344 and LDQ10, labeled with fluorescein, detect membrane mucins expressed in bladder cancer cells, mainly those of a low grade (Fradet et al, 1997; Mian et al, 1999). To date, the diagnostic value of uCyt+ has not been validated in a study with a large number of patients.

Accordingly, the goal of our study was (a) to determine the diagnostic accuracy of the uCyt+ assay in a prospective multicenter protocol, including new cases and cases followed after TUR, and (b) to discuss its hypothetical widespread applicability for monitoring bladder cancer, whether or not combined with urinary cytology.

Results

In 52 cases (6.9%), samples were excluded from the analysis for various reasons, leading to a total of 694 valid cases. The study population was composed of 144 women and 550 men, 32 to 92 years old (mean age, 66.2 ± 12.8 years).

The cases studied were distributed into two groups: (1) in 236 cases (34.0%), patients were referred to the urologist for symptoms suggesting bladder cancer, and (2) in 458 cases (66.0%), patients were followed after TUR for superficial bladder tumor in 455 cases, or for urothelial carcinoma of the upper urinary tract in 3 cases. TUR was performed at least 6 months previously (mean 34 ± 25 weeks), and bacillus Calmette-Guérin (BCG) immunotherapy, when applicable, was performed at least 1 month previously in every case.

In new patients with symptoms possibly indicating bladder cancer (Table 1), there were 66 positive cystoscopy findings at bladder level at the date of consultation (27.9%). All of them had biopsies or TUR except one patient not reviewed. Thirteen patients had suspicious mucosal abnormalities, 12 of whom (92.3%) having negative biopsy results. One urothelial carcinoma was diagnosed at the upper urinary tract level.

Cystoscopy was negative in 156 cases, 12 of which having negative random biopsies. Putting aside three nonurothelial tumors, there remained 59 new urothelial carcinomas diagnosed by histopathology (including the case of upper urinary tract tumor).

In the 458 patients followed after TUR for bladder cancer, recurrence was suspected by positive or suspicious cystoscopy in 105 cases (22.9%) at the date of consultation (Table 2). Two urothelial carcinomas were diagnosed at the ureteral level. Cystoscopy was negative in 351 cases (76.6%); random biopsies performed in 15 cases were positive in only two patients. Globally, 87 recurrent urothelial carcinomas were diagnosed histologically (including the two upper urinary tract tumors).

Table 1. Clinical Data in the 236 New Symptomatic Patients

Characteristic	no.	%
Symptoms		
Gross hematuria	148	62.7
Dysuria, pollakiuria	27	11.4
Cystalgia	17	7.2
Cystitis, urinary infection	13	5.5
Microhematuria	9	3.8
Other conditions	8	3.4
Missing data	14	5.9
Positive cystoscopy at consultation		
Negative TUR	5	2.1
Positive TUR	60[a]	25.4
TUR not performed	1[b]	0.4
Suspicious cystoscopy at consultation		
Negative biopsy	12	5.1
Positive biopsy	1	0.4
Negative cystoscopy at consultation		
Negative biopsy	12	5.1
Biopsy not performed	144	61.0
Upper urinary tract tumor	1	0.4

TUR, transurethral resection.
[a] Includes three nonurothelial tumors (two prostatic adenocarcinomas invading the bladder wall and one squamous cell carcinoma of the bladder).
[b] Patient not reviewed.

Global Efficiency of Tests

In the whole series, there were 161 positive or suspicious urinary cytology results, whereas 190 uCyt+ assays were found positive. Tests were simultaneously positive in 101 cases (14.5%). Urinary cytology was considered unsatisfactory for evaluation (too low cellularity or hardly recognizable cells) in only 21 cases (3%). Fluorescence immunocytochemistry was unsatisfactory for evaluation in only 13 cases (1.8%) due to an insufficient number of cells (<500 per smear).

Sensitivity Calculation

Globally, 210 of 694 patients (30.2%) were subjected to biopsies, TUR, or surgery for upper urinary tract tumor at the entry. Various types of tumors were found in 150 cases, including a Mostofi's papilloma in one case, which was considered negative. Three nonurothelial tumors (two prostatic adenocarcinomas and one epidermoid carcinoma) were excluded from calculations, leading to a total of 146 urothelial carcinomas (Table 3).

In new patients, sensitivity of fluorescence immunocytochemistry was 40%, 88.2%, and 76.7%, whereas

Table 2. Pathologic History and Findings at Consultation and Histopathologic Data in 458 Patients after TUR

Characteristic	no.	%
History of patients treated by TUR for bladder tumor		
pTa G1–2	236	51.5
pTa G3 and pTa G2 + CIS	37	8.1
pTa GX (grade not determined)	3	0.6
pT1 G1–2	36	7.9
pT1 G3 and pT1 G2 + CIS	92	20.1
≥ pT2	24	5.2
pTIS	15	3.3
pTX (stage not determined)	8	1.7
Epidermoid carcinoma	3	0.6
Tubular adenoma	1	0.2
History of patients treated for upper urinary tract tumor		
pTa G2	1	0.2
pT1 G3	1	0.2
pTX G2	1	0.2
History of patients according to grade		
Grade 1	132	28.8
Grade 2	143	31.2
Grade 3	169	36.9
Grade not recorded (GX)	10	2.2
Nonurothelial tumor	4	0.8
Positive cystoscopy at consultation		
Negative TUR	6	1.3
Positive TUR (urothelial carcinoma)	77[a]	16.8
Mostofi's papilloma	1	0.2
TUR not performed	3[b]	0.6
Suspicious cystoscopy at consultation		
Negative biopsy	12	2.6
Positive biopsy (urothelial carcinoma)	6	1.3
Negative cystoscopy at consultation		
Negative biopsy	13	2.8
Positive biopsy	2	0.4
Biopsy not performed	336	73.4
Upper urinary tract tumor	2	0.4

TUR, transurethral resection.
[a] Includes two fulgurated tumors considered pTa G1 urothelial carcinomas.
[b] Patients not reviewed.

that of urinary cytology was 30%, 70.6%, and 83.3%, respectively, in newly diagnosed G1, G2, and G3 bladder tumors, respectively (χ^2 Yates = 0.64, ns). When assays were combined, values reached 50%, 94.1%, and 93.3%, respectively, and were significantly higher than those of urinary cytology only ($\chi^2 = 3.9$, $p < 0.05$).

In patients followed after TUR, the sensitivity of fluorescence immunocytochemistry was 61.9%, 66.7%, and 76.9%, whereas that of urinary cytology was 38.1%, 58.3%, and 64.1%, respectively, in newly diagnosed G1, G2, and G3 bladder tumors, respectively (χ^2 Yates = 4.65, $p < 0.05$). When assays were combined, sensitivity values were increased to 61.9%, 79.2%, and 87.2%, respectively, and were much higher than those of urinary cytology only ($\chi^2 = 11.3$, $p < 0.001$).

As expected, sensitivity of urinary cytology in detecting G3 lesions was lower in follow-up cases than in newly diagnosed cases (64.1% versus 83.3%, $\chi^2 = 3.87$, $p < 0.05$), whereas values obtained with uCyt+ were nearly identical (76.9% and 76.7%).

Specificity Calculation

In both categories of patients, there were 509 negative cystoscopy findings at the date of consultation: 156 of 236 new cases (66.1%) and 353 of 458 follow-up cases (77.0%), among which 27 (5.3%) had random biopsies. Two biopsy samples were found positive and were excluded from calculation; one was a recurrent pTa G1 tumor followed after pTa G1 for 4.5 years. It was falsely negative with both urinary cytology and fluorescence immunocytochemistry. The other case was a new pT3 G3 tumor followed after pT1 G3 for 5 years. It was found positive with both tests. Additionally, two pTX GX tumors found at the upper urinary tract level were excluded from calculation, thus allowing 505 cases to be used for specificity calculation (Table 4).

In patients consulting for symptoms, uCyt+ and urinary cytology were negative in 130 of 156 cases with negative cystoscopy findings (specificity = 83.3%). In patients followed for urothelial carcinoma of the bladder, fluorescence immunocytochemistry was negative in 286 of 349 negative cystoscopy cases (specificity = 81.9%), whereas urinary cytology was negative in 301 of 349 cases (specificity = 86.2%).

Urinary cytology was found positive or suspicious in 61 of 505 negative cystoscopy cases (12%). Histopathologic control was obtained in five cases: three being negative, one being a pTX GX upper urinary tract tumor with positive uCyt+ test, and one being a pT3 G3 bladder tumor positive with both tests. Putting aside these two tumor cases, there remained 59 of 505 (11.7%) falsely positive urinary cytology samples.

Fluorescence immunocytochemistry was positive in 80 cases with negative cystoscopy. Random biopsies were performed in five cases: four were negative, and one was a bladder pT3 G3 urothelial carcinoma. Additionally, two pTX GX tumors were found at the upper urinary tract level; three cases had to be excluded from calculation. Accordingly, the false-positive rate of the uCyt+ assay was 77 of 505 cases (15.2%).

Follow-Up Data

To test whether positive uCyt+ results found in patients with negative cystoscopy were false-positive or were predictive for tumor recurrence, we collected the cystoscopy and histopathology data. When applicable these data were obtained 6 and 12 months after inclusion. Follow-up data could be obtained in 17 patients with negative cystoscopy but positive uCyt+ test and in 59 patients taken as reference in whom both controls were simultaneously negative.

At 6 months, the recurrence rate in patients with positive uCyt+ test was not statistically different from

Table 3. Sensitivity of Urinary Cytology and uCyt+ in Urothelial Carcinomas, Including the Upper Urinary Tract

Characteristic	*N*	Positive urinary cytology	Se (%)	Positive uCyt+ test	Se (%)	Se of combined assays (%)
Newly diagnosed tumors						
pTa G1–2	22	10	45.4	15	68.2	16/22 (72.7)
pTa-1 G3	14	13	92.8	11	78.6	14/14 (100.0)
pT1 G1–2	4	4	100.0	3	75.0	4/4 (100.0)
≥ pT2	11	9	81.8	8	72.7	9/11 (81.8)
pTIS	1	0	0.0	1	100.0	1/1 (100.0)
≥ pTa + CIS	5	4	80.0	4	80.0	5/5 (100.0)
pTX GX (bladder)	1	1	100.0	1	100.0	1/1 (100.0)
pTX GX (UUT)	1	1	100.0	1	100.0	1/1 (100.0)
Grade 1	10	3	30.0	4	40.0	5/10 (50.0)
Grade 2	17	12	70.6	15	88.2	16/17 (94.1)
Grade 3	30	25	83.3	23	76.7	28/30 (93.3)
Grade not recorded (GX)	2	2	100.0	2	100.0	2/2 (100.0)
Total	59	42	71.2	44	74.6	51/59 (86.4)
Recurrent tumors						
pTa G1–2	42	19	45.2	27	64.3	30/42 (71.4)
pTa-1 G3 and pTX G3	18	14	77.8	16	88.9	16/18 (88.9)
pT1 G2	3	3	100.0	2	66.7	3/3 (100.0)
≥ pT2	16	9	56.2	10	62.5	13/16 (81.2)
pTIS	2	2	100.0	1	50.0	2/2 (100.0)
≥ pTa + CIS	3	0	0.0	3	100.0	3/3 (100.0)
pTX GX (bladder)	1	ns	–	1	100.0	1/1 (100.0)
pTX GX (UUT)	2	1	50.0	2	100.0	2/2 (100.0)
Grade 1	21	8	38.1	13	61.9	13/21 (61.9)
Grade 2	24	14	58.3	16	66.7	19/24 (79.2)
Grade 3	39	25	64.1	30	76.9	34/39 (87.2)
Grade not recorded (GX)	3	1	33.3	3	100.0	3/3 (100.0)
Total	87	48	55.2	62	71.3	69/87 (79.3)

Se, sensitivity; UUT, upper urinary tract.

Table 4. FP and Sp of Assays in Patients with Negative Cystoscopy Findings

Characteristic	*N*	Positive UC	FP (%)	Negative UC	Sp (%)	Positive IC	FP (%)	Negative IC	Sp (%)
New symptomatic patients									
No biopsy	144	15		121 (8 us)		20		120 (4 us)	
Negative biopsies	12	0		9 (3 us)		1		10 (1 us)	
Total	156	15	9.6	130 (11 us)	83.3	21	13.5	130 (5 us)	83.3
Follow-up cases									
No biopsy	336	41		291 (4 us)		52		275 (7 us)	
Negative biopsies	13	3		10		4		11	
Total	349	44	12.6	301 (4 us)	86.2	56	16.0	286 (7 us)	81.9
Whole population	505	59	11.7	431 (15 us)	85.3	77	15.2	416 (12 us)	82.4

FP, false-positivity; IC, immunocytochemistry; Sp, specificity; UC, urinary cytology; us, unsatisfactory for evaluation.

in the reference population (35.7% versus 13.9%, χ^2 Yates = 1.95, ns). Conversely, at 1 year, 8 of 17 patients (47%) with negative cystoscopy but positive uCyt+ test had recurred versus 7 of 59 patients (11.9%, χ^2 Yates = 8.22, $p < 0.01$) with a negative assay, thus indicating that a positive uCyt+ test has a strong predictive value for tumor recurrence at 1 year.

Discussion

Voided urinary cytology has been used since 1945 as the only available noninvasive test for monitoring bladder cancer, but it is limited by both observer subjectivity and low sensitivity in detecting low grade tumors, and sometimes yields false-negativity in high grade urothelial carcinomas.

On the other hand, cystoscopy is invasive, time-consuming, and rather expensive. It may also be inconclusive in some conditions. For example, in patients with an indwelling catheter or inflammation in course, cystoscopy may not be conclusive due to grossly abnormal appearance of the urothelial mucosa. In some cases, carcinoma in situ (pTIS tumors)

can only be detected by biopsies in zones where mucosa appears almost normal. In such cases, urinary cytology is generally positive and shows high grade tumor cells, but it may be falsely negative. Hence, none of these tests is ideal for screening for bladder cancer. Despite its limitations, cystoscopy aided by urinary cytology remains the mainstay for diagnosing and monitoring bladder cancer to date (Saad et al, 2001).

The high propensity of urothelial carcinomas for tumor recurrence makes necessary prolonged periods of follow up and frequent consultations for each patient treated after bladder cancer. The deficiencies of currently available tests render necessary but suboptimal the methods used for tumor surveillance. It is for those reasons that for years both urologists and pathologists have tried to broaden the scope of the procedures following TUR for bladder tumor. Noninvasive methods that could replace time-consuming and tedious cystoscopy controls, which sometimes induce nosocomial infections (Clark and Higgs, 1990), would be useful in routine urology practice.

The sensitivity of urinary cytology in detecting grade-1 and grade-2 urothelial carcinoma is 15% to 30% in most series (Bastacky et al, 1999). Despite its high sensitivity in grade-3 tumors, the inaccuracy of urinary cytology in detecting recurrences of most bladder tumors has stimulated for many years the search for new noninvasive markers.

DNA flow cytometry does not have a higher sensitivity than that of urinary cytology (Gourlay et al, 1995). Despite promising preliminary results, diagnostic tests such as telomerase activity detection or cytokeratin 20 have shown to be technically complicated, and their mean sensitivity is no higher than that of urinary cytology (Ramakumar et al, 1999). Moreover, these tests are not specific for malignancy (Cassel et al, 2001; Konety and Getzenberg, 2001). Other assays such as Bard BTA Stat test and NMP22 assay have shown a 67% and 70% sensitivity, respectively, with approximately 70% specificity for both tests (Giannopoulos et al, 2001; Sarosdy et al, 1997). BTA Stat test and telomerase activity seem more sensitive than urinary cytology in detecting G1 and G2 tumors. Microsatellite detection studies have shown loss of heterozygoty, particularly on chromosome 9 or other alterations in microsatellites with a 85% to 95% sensitivity and about 95% specificity (Lotan and Roehrborn, 2003). A widespread applicability of microsatellite analysis would need automation of the assay, thus rendering the detection process accessible for less specialized institutions.

Other studies have been devoted for years to cell cycle markers that could aid in monitoring bladder tumors but none of them has proven its clinical utility (Hölmang et al, 2001; Ponsky et al, 2001; Sanchez-Carbayo et al, 2001).

In comparison with the above-mentioned assays that show insufficient sensitivity, lack specificity for cancer, or demonstrate serious technical constraints, interphase fluorescence in situ hybridization (FISH) and fluorescence immunocytochemistry using the uCyt+ assay (formerly ImmunoCyt, DiagnoCure) appear as the two most promising tests.

The UroVysion FISH assay (Vysis Inc., Downers Grove, Illinois) is based on directly labeled fluorescent probes to the pericentromeric regions of chromosomes 3, 7, and 17 and on a probe to the 9p21 band. In a multicentric approach, overall sensitivity values of 71%, 50%, and 26% were obtained for FISH, BTA Stat test, and urinary cytology, respectively. FISH was negative, with a specificity of 95% in healthy volunteers or patients with no history of urothelial carcinoma, even in patients with inflammatory conditions or in those treated with BCG (Halling et al, 2000; Sarosdy et al, 2002). Further clinical evaluation is needed, owing to the high specificity of FISH, which could reduce the number of invasive tests performed that yield positive results in the absence of disease (Sarosdy et al, 2002).

The first clinical study concerning uCyt+ in bladder cancer monitoring included data obtained in 102 asymptomatic controls and 198 patients with bladder cancer (Fradet et al, 1997). The diagnostic value of urinary cytology was significantly higher when combined with the uCyt+ assay, reaching 96.4% of pTa tumors, but it depended on the cut-off level chosen for positive results.

Additional data were provided in 264 consecutive patients, including 150 follow-up cases (Mian et al, 1999). Fluorescence immunocytochemistry combined with urinary cytology had an 89.9% sensitivity overall, whereas uCyt+ only was 86.1% sensitive and 79.4% specific for bladder cancer. The overall sensitivity value of urinary cytology was only 46.8%, as mentioned in many studies in which urinary cytology is near 30% sensitivity in low grade tumors. As found in the Fradet series (Fradet et al, 1997) the percentage of uCyt+ assays unsatisfactory for evaluation was $< 7\%$.

Four more recent series included < 120 patients and showed results varying between 45% and 100% sensitivity (Bunting et al, 2000; Lodde et al, 2001; Olsson and Zackrisson, 2001; Vriesema et al, 2001). A more recently published series included 235 patients and 102 cancer cases (Lodde et al, 2003). Sensitivity values of 78% to 85% and 90% to 100% were obtained for G1 and G2 to G3 tumors, respectively, with uCyt+ and urinary cytology combined.

The present series included 694 patients in a multicenter protocol, including cystoscopy, urinary cytology, and histopathology as conventional means for follow up without specific requirements for urinary cytology. Each pathologist concerned, following recommendations of the WHO/ISUP 1998 classification (Epstein et al, 1998), rendered positive, suspicious, or negative results as described in the Materials and Methods section below. Fluorescence immunocytochemistry was performed independently of the cytologic and histopathologic results.

Our results concerning urinary cytology are available online with other reports. Sensitivity results are not higher than those usually reported but demonstrate that sensitivity for detecting G3 tumors is sig-

nificantly lower in patients treated after TUR for bladder cancer, thus reinforcing the hypothesis that when G3 induces muscle infiltration, normal urothelium that reconstitutes in surface hides recognizable tumor cells.

In such cases, only tests based on specific tumor antigens or markers can increase the sensitivity of urinary cytology. This is demonstrated in this study in which the combination of urinary cytology with fluorescence immunocytochemistry increases the value of urinary cytology from 55.2% to 79.3% in the diagnosis of recurrent tumors. Regarding uCyt+, the data shown demonstrate that this new technique is associated with higher sensitivity than conventional urinary cytology and that this increase is not linked to a dramatic decrease in specificity.

We demonstrate that in all cases, fluorescence immunocytochemistry increases the diagnostic value of urine samples and that better results are obtained by combining the assays. The combination allowed 86.4% and 79.3% sensitivity, respectively, in new symptomatic patients and in patients followed after TUR.

Concerning specificity in cases with negative cystoscopy, the 13.5% and 16% positive uCyt+ results, respectively, in new patients and in patients previously treated by TUR needed to be controlled by follow up because 28% of positive uCyt+ tests may have a recurrence within 3 to 6 months (Lodde et al, 1999). In the present series, a high predictive value for tumor recurrence is associated with positive uCyt+ results in patients with negative cystoscopy (47% at 1 year versus 11.9% in a control group).

The uCyt+ technique appears easy to perform, provided recommendations are carefully followed and provided fluorescence equipment is of good quality. Observer subjectivity is reduced to interpretation of low levels of green fluorescence, and difficulties can easily be surmounted by training and systematic comparison with controls. Moreover, uCyt+ data can simply be expressed as positive or negative for tumor cells. With regard to the lack of insuperable constraints concerning both urinary cytology and fluorescence immunocytochemistry, immediate applicability of assays is conceivable in routine histopathology laboratories.

The combination of uCyt+ with urinary cytology is particularly useful as an adjunct to cystoscopy and could replace cystoscopy itself in the follow up of selected patients. The combined use of tests might prolong the interval of cystoscopy in patients monitored for bladder cancer recurrence, particularly in the pTa G1–G2 category. This would significantly reduce the cost to patients who, by current standards, undergo cystoscopy every 3 months for the first 1 to 2 years following TUR. The risk of invasion of low grade lesions being low, and provided almost all potentially invasive, high grade lesions are detected, such a protocol would be acceptable.

We conclude that combining uCyt+ to urinary cytology improves the detection of urothelial carcinomas as well in patients suspicious for bladder cancer as in those followed after TUR. Using these tests would reduce the frequency of cystoscopy in selected categories of patients.

Materials and Methods

Protocol Design and Population Studied

The study was performed in 10 Departments of Urology and 9 Departments of Pathology in France. During a 9-month period, 746 voided urine samples collected before cystoscopy were obtained from 746 consecutive patients.

Samples were obtained from male and female patients at least 18 years old, either attending a consultation in a urology department for symptoms possibly indicating bladder cancer (gross hematuria, microscopic hematuria, recurring cystitis, or mictionary disorders) or being followed after complete TUR or BCG immunotherapy for urothelial carcinoma at least 1 month previously.

Samples from patients currently being treated with BCG immunotherapy, those having had TUR or electroresection in the month preceding the control, and those with known urinary infection or lithiasis were excluded. Smoking status of the patients and prior exposition to bladder carcinogens were not recorded.

The patients were evaluated by conventional means, including cystoscopy and urinary cytology combined. The results of cystoscopy were recorded in each case as positive (mainly papillary growth), suspicious (irregular appearance in flat mucosa), or negative. A TUR was performed in every case of papillary bladder lesion, and mucosal abnormalities suspicious for carcinoma in situ were evaluated by biopsies.

Histopathologic characterization and grading used was that of the WHO/ISUP 1998 classification (Epstein et al, 1998), and the staging was performed according to the UICC TNM classification. A small number of tumors were directly fulgurated by the urologist and had no histology, considering that the lesion was small and apparently superficial.

Collection of Specimens

At least 40 ml of voided urine (excluding the first-morning urine) was taken before cystoscopy to avoid interference of the anesthetic gel with the uCyt+ assay. Samples were immediately fixed with an equal volume of 50% ethanol, stored at 4° C and sent to the laboratory within 24 hours. After homogeneization, half of the volume was used for conventional cytology and half was prepared for fluorescence immunocytochemistry.

Fluorescence Immunocytochemistry (uCyt+ Assay)

Upon receipt of the sample, 1 ml of fixative buffered solution was added to the urine, and the sample was incubated at room temperature for 15 minutes. The urine was then filtered through a polycarbonate membrane with a porosity of 8 μm in a device connected to a 50-ml syringe. Cells captured on the filter were

carefully blotted on silanized slides and immediately fixed with 50% isopropanol.

Slides were rehydrated through 80%, 70%, and 50% ethanol and rinsed in distilled water. Cells were then stained with a solution of Harris Hematoxylin containing 4% acetic acid for 90 seconds. After washing in distilled water, a blocking solution was applied for 15 minutes at room temperature in a dark humid chamber. After draining, the slide was incubated with the monoclonal antibodies for 1 hour at room temperature and rinsed twice in a PBS solution containing 0.5% Tween 20. Another rinse in PBS was applied to the slide, which was finally mounted with Permafluor.

Negative controls were prepared from the line Luci-6 issued from human lung large cell carcinoma tumor cells. Cultured cells were suspended in formaldehyde/ethanol and preserved at 4° C until their use as control. Positive controls were prepared from the human bladder tumor line TMGHU-3 injected to nude mice. Tumor cells were extracted from subcutaneous tumors, mechanically dissociated, suspended in formaldehyde/ethanol, and preserved at 4° C until their use as control. Negative and positive controls were systematically included in each series of labeling.

Slides were stored at 4° C for up to 7 days before reading under an epifluorescence microscope equipped with a double filter for FITC and Texas Red emission.

The red fluorescence showed the presence of high molecular weight glycosylated carcinoembryonic antigen, whereas the green fluorescence revealed cells positive for tumor-related mucins. A sample was considered positive when at least one clearly fluorescent cell was noted, whatever its intensity (x to xxx according to a set of color pictures provided with manufacturer specifications).

Usually a single pathologist read the specimens in each center. High levels of fluorescence (xx to xxx) were easily recognized. Low levels of green fluorescence (x) needed careful comparison with positive and negative controls. Conversely, the criteria retained for red fluorescence was easily recognized whatever the intensity (sparkling grains on the cell membrane). The samples were considered "unsatisfactory for evaluation" in cases with low cellularity (<500 cells per slide).

Conventional Cytology

Slides for conventional cytology were prepared by centrifugation followed by cytocentrifugation or smearing. Cells were stained according to the standard Papanicolaou procedure. Specimens were analyzed without knowledge of the uCyt+ assay results and blind to the histopathologic results as well.

Cytologic results were categorized as positive or negative for tumor cells, whatever their grade. Normal, inflammatory, reactive, and degenerative conditions of the urothelial component, as described by previous studies (Murphy, 1990; Koss, 1995) were considered negative, as well as urothelial atypias of undetermined significance. Specimens in which neoplastic cells were recognized (grade 3 and grade 2 clearly identified in the pathology report, strong suspicion for papillary low grade tumors based on architectural and nuclear abnormalities, as described elsewhere) were considered positive for urothelial carcinoma (Murphy, 1990).

Specimens containing atypical cells but no actual evidence for malignancy were considered "suspicious for cancer" and were included in the positive ones. Specimens with very low cellularity or hardly recognizable cells were considered "unsatisfactory for evaluation" and were excluded from calculations.

Analysis of Data

Assuming that cystoscopy is better suited for papillary lesions of the bladder and that urinary cytology is most effective when high grade lesions (pTaG3, pT1G3, and pTIS) are present, we used positive cystoscopy confirmed by histology as the gold standard for calculating sensitivity of uCyt+ assay and urinary cytology.

However, we considered that the rate of false-negative results would be different in the two populations studied. In patients followed after several TURs or partial cystectomy, infiltrative high grade lesions (pT2G3, pT3, or more) can be missed by urinary cytology simply because a normal urothelium reconstitutes in surface. In such cases, urinary cytology is falsely negative, whereas bladder tumor antigens could be evidenced by other methods (Ozen and Hall, 2000; Kausch and Bohle, 2001). Conversely, in new patients in whom a great majority of lesions are superficial at presentation, urinary cytology would give better results than in follow up, especially for detecting G3 tumors. For testing these hypotheses, we analyzed data by systematically comparing the two clinical situations (newly diagnosed and chronic).

In calculating specificity of fluorescence immunocytochemistry and urinary cytology, we considered that negativity for urothelial carcinoma was better addressed when cystoscopy was negative (cases initially classified as suspicious but without histologic control have been reclassified as negatives), thus placing the observer in the most common practice of urology. The value of combined assays can be calculated when at least one of the two tests (uCyt+ and urinary cytology) is positive.

Frequency data were analyzed using chi-square tests with Yates correction for paired series when applicable. A probability level of 0.05 was regarded as significant. Epidermoid carcinomas, adenocarcinomas, and prostatic tumors were not included in the statistics.

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