

# The diagnostic value of cytohistological urine analysis and cytokeratin 20 in malignant and atypical urothelial cells

S. NEGRI<sup>1</sup>, P. BIAVATTI<sup>2</sup>, A. BONDI<sup>1</sup>

<sup>1</sup>Pathological Anatomy, Bologna Health Local Sanitary Unit, Maggiore Hospital, Bologna, Italy; <sup>2</sup>Epidemiology, Health Promotion and Risk Communication, Bologna Health Local Sanitary Unit, San Giorgio di Piano Hospital, Bologna, Italy

## Key words

Bladder cancer • Cytokeratin 20 • Urine cytology • Atypical urothelial cells

## Summary

**Introduction.** To determine the ability of cytohistology and cytokeratin 20 (CK 20) expression in malignant and atypical cells (AUC) from urine to serve as a diagnostic tool for assessing urothelial carcinoma (UC).

**Methods.** Diagnoses from 55 urine cytological samples from 55 patients were analyzed and correlated with subsequent biopsy findings. A total of 50 archived urine slides from patients that received a cytological diagnosis and histological follow-up were selected for immunostaining with monoclonal CK 20 antibodies and elaborated by Z-test for proportions.

**Results.** The majority of all positive or atypical smears (24; 89%) were confirmed through histological analysis. The majority of uri-

nary cytological diagnoses reported as negative (15; 54%) were also confirmed through biopsies. The overall sensitivity, specificity, PPV, and NPV were 65%, 83%, 89%, and 54%, respectively. All 13 smears cytologically determined to contain malignant cells, with subsequent biopsies confirming UC, exhibited strong positive staining with the CK 20 antibody. All cases evaluated as benign both cytologically and histologically had negative CK 20 staining. Of the 15 AUC cases with lesions confirmed through biopsies, 11 (73%) had atypical cells that stained positive for CK 20.

**Discussion.** Our results demonstrate the diagnostic value of urinary cytology and confirm CK 20 as an adjunct marker for the diagnosis of UC and for the triage of AUC.

## Introduction

Urothelial bladder cancer is the seventh most common cancer worldwide in men (10.1 new cases per 100,000 people each year) and the 17th most common cancer in women (2.5 new cases per 100,000 people each year). High incidence rates are observed in developed countries. In Italy, when considering men and women together, bladder cancer is the third most common cancer (after breast and colorectal cancers) <sup>1</sup>. Cytological analysis of voided urine has a high sensitivity in the diagnosis of high grade tumors, including flat carcinoma *in situ*, but has less sensitivity for the recognition of low-grade papillary tumors <sup>1,2</sup>. However, due to its high specificity, non-invasiveness, simplicity, and familiarity, cytological methods are still very reliable for the detection, diagnosis, and follow-up of bladder tumors and other anatomic components of the lower urinary tract, especially when

coupled with cytoscopic examination <sup>3</sup>.

The cytological detection of urinary bladder neoplasms is limited by many factors, including the classification used for correlation, cytomorphological constraints, specimen quality, and the interpretive experience and skill of the pathologist <sup>4</sup>. Brimo et al. <sup>5</sup> reported that the Papanicolaou Society of Cytopathology, in an attempt to standardize the diagnostic categories, recommended in 2004 that a diagnostic scheme include an “atypical urothelial cells” category, followed by a comment to describe differential diagnostic possibilities (reactive versus malignant) <sup>6</sup>. However, the criteria that should be used to separate reactive atypia from neoplastic atypia were not clearly defined in that article or in the literature. Raab <sup>7</sup> has suggested lowering the proportion of atypia diagnosed by shifting diagnosis to more definitive categories using molecular testing to increase diagnostic accuracy.

## Correspondence

Silvana Negri, U.O. Anatomia, Istologia Patologica e Citodiagnostica, Ospedale Maggiore, Via Dell’Ospedale 8, 40133 Bologna, Italy - Tel.: +39 051 6478110 - Fax: +39 051 6478660 - E-mail: silvana.negri@ausl.bologna.it

However, other quality improvement methods, such as specimen re-preparation, panel review, and education were also listed as approaches potentially more effective for classifying cases with atypia.

If a urinary specimen contains neoplastic cells poorly preserved or morphologically indistinguishable but genetically/antigenically distinct from reactive cells, it can be detected by one or more ancillary techniques<sup>2</sup>. The expression of CK 20, found through histological immunostaining, has proven useful for recognizing dysplasia, high-grade flat carcinomas, and low-grade and high-grade papillary urothelial carcinomas<sup>8-11</sup>. Further data has even suggested its potential for predicting the biological behavior of papillary urothelial pTa and pT1 neoplasms, and in grading papillary urothelial tumors<sup>12-15</sup>. Many authors have also demonstrated its value as a simple and non-invasive cytological method useful for the detection of urothelial carcinoma<sup>16-21</sup>. Thus, in this study we analyzed the diagnostic value of urinary cytology in daily clinical practice and evaluated the diagnostic potential of CK 20 expression in malignant and atypical cells from voided urine.

## Methods

### COLLECTION AND TREATMENT OF SAMPLES

Urine samples were collected from patients at Maggiore Hospital, Bologna for three consecutive days in cups containing an alcohol-based fixative. The three samples were returned to the hospital, mixed together, filtered, and collected on a single slide. The sources of urinary specimens included inpatients from our hospital services and outpatients from general medicine services, mainly with the following indications: screening for urothelial neoplasms in patients with hematuria or irritative urinary symptoms, and the surveillance of patients with a history of urothelial neoplasia. Cytological results were obtained from 55 patients as part of our daily routine practice during the first semester of the year 2011. These patients had biopsies performed within 6 months after a cytological test and were placed into one of the following diagnostic categories: unsatisfactory for evaluation (UE), negative for abnormalities in epithelial cells (N), atypical urothelial cells (AUC), malignant (CA) (including high-grade urothelial carcinoma, low-grade urothelial carcinoma, and suspicious cases). Based on morphological studies suggesting that cell clusters in voided specimens are associated with higher rates of cancer on histological follow-up<sup>22</sup>, and observations by Koss<sup>23</sup>, we restricted a diagnosis of AUC to papillary cell clusters or single cells characterized by nuclear enlargement and hyperchromasia (without the coarse granularity of chromatin, abnormalities of nuclear contour, or large nucleoli characteristic of cancer cells). Cells with severe cytological and architectural changes potentially pre-neoplastic, yet falling short of the diagnostic threshold for transitional cell carcinoma, were categorized as suspicious. Cytological results were correlated with their

subsequent histological diagnoses. Cytohistological correlation was patient-based rather than specimen-based, such that a patient with multiple specimen correlations was only tabulated one time. The sensitivity, specificity, and predictive values (positive and negative) were calculated overall and according to tumor grade.

### Immunocytochemistry

A total of 50 consecutive urinary cytological slides prepared during the year 2011 corresponding to patients from four risk groups on the basis of their original cytological diagnosis (negative for epithelial abnormality, AUC, or positive for urothelial carcinoma) and clinical outcome (presence or absence of urothelial carcinoma) were retrieved from the archive and selected for immunocytochemistry. Papanicolaou-stained slides were initially kept overnight in xylene to remove the coverslip and mounting media. The smear was then re-hydrated with subsequent distilled water (DI) rinses before being loaded on the IHC platform (Ventana BenchMark Ultra, Roche Tissue Diagnostics, Rotkreuz, Switzerland). The validated protocol requested an online mild heat pre-treatment at pH 8,2 AR buffer for 36 min at 95°C (Ultra CC1 = Tris Borato EDTA Ph 8,2 \_ RTU, Roche Tissue Diagnostics, Rotkreuz, Switzerland) to retrieve antigens. After a conventional online endogenous peroxidase inhibition step, slides were incubated with an RTU monoclonal antibody for CK 20 (clone SP33) for 24 min at 36°C (Confirm anti-CK 20, Roche Tissue Diagnostics, Rotkreuz, Switzerland), detected with the Ventana multimeric DAB detection Kit (Ultraview DAB, Roche Tissue Diagnostics, Rotkreuz, Switzerland) and finally counterstained with Mayer hematoxylin for 8 min at 37°C (Hematoxylin II, Roche Tissue Diagnostics, Rotkreuz, Switzerland). A diamond pencil was used on the reverse side of the glass to mark atypical or malignant cells present in Papanicolaou-stained smears before the CK 20 immunocytochemistry session. The strength of cytoplasmic CK 20 staining was measured by two independent cytologists blind to the final histological diagnoses. The statistical Z-test for proportions was performed with SPSS software to test the significance of difference of CK 20 positivity between two groups: one group with AUC and subsequent biopsy-confirmed lesions, and one group with AUC and subsequent negative biopsy results.

## Results

Cytohistological correlation results are summarized in Table I. Positive or atypical results (at least one urinary cytology) was found in 27 patients (13 and 14, respectively). Of these, 24 (89%) were confirmed histologically, while 3 (11%) were negative. Urinary cytology was negative in 28 patients, 15 confirmed by biopsy (54%), while 13 (46%) had at least one positive histological evaluation. The overall sensitivity, specificity, PPV, and NPV were 65%, 83%, 89%, and 54%, respectively. Of the 35 UC found upon biopsy, 13 were missed by cytology (37%), including 9 (69%) LG UC. The charac-

**Tab. I.** Cytologic-histologic correlation according to histologic tumor grade.

		Biopsies				Total
		HG UC	LG UC	Atypical	Negative	
Cytology	CA	12	1	–	–	13
	AUC	4	5	2	3	14
	N	4	9	–	15	28
Total		20	15	2	18	55

HG UC, high-grade urothelial carcinoma; LG UC, low-grade urothelial carcinoma; CA, malignant; AUC, atypical urothelial cells; N = negative for epithelial abnormality.

Sensitivity = 65%, specificity = 83%, PPV = 89%, NPV = 54%

**Tab. II.** Original cytologic and histologic follow-up of 50 study patients for CK 20 analysis.

Patient groups	Cytology	Biopsies				Total
		HG UC	LG UC	Atypical	Negative	
1	CA	12	1	–	–	13
2	AUC	5	8	2	0	15
3	AUC	0	0	0	7	7
4	N	0	0	0	15	15
Total		17	9	2	22	50

HG UC, high grade urothelial carcinoma; LG UC, low grade urothelial carcinoma; CA, malignant; AUC, atypical urothelial cells; N, negative for epithelial abnormality.

**Tab. III.** CK 20 expression from urine cytology slides.

Patients groups	Cytology	No. of slides	CK 20 negative slides	% number of slides with negative CK 20 staining	CK 20 positive slides	% number of slides with positive CK 20 staining
1	CA	13	0	0	13	100
2	AUC *	15	4	26.7	11	73.3
3	AUC *	7	6	85.7	1	14.3
4	N	15	15	100	0	0

CA, malignant; AUC, atypical urothelial cells; N, negative for epithelial abnormality

\*Difference in CK 20 positivity; significance:  $P < 0.05$  by Z-test for proportions.

teristics of urinary specimens in patients with histologically-confirmed bladder neoplasm were the following: histologically-confirmed HG UC occurred in 20 patients (57%); 12 of these (60%) had their neoplasm detected by urinary cytology and 4 had a previous AUC diagnosis. At biopsy, LG UC involved 15 patients (43%), with 6 of these (40%) having had a previous malignancy or AUC diagnosis (1 and 5, respectively). The distribution of cytological features within the subgroups of atypical urine cytology were the following: 9 (64%) had a positive follow-up biopsy (4 HG UC; 5 LG UC), 2 were atypical at biopsy as well, while 3 (21%) were negative at the subsequent biopsy.

Table II lists all 50 patients in the CK 20 study, including their original urinary cytology and histological diagnosis. Group 1 consisted of all 13 cases of malignant cytological diagnoses and biopsy-confirmed UC (12 HG UC, 1 LG UC). Group 4 had a negative urinary cytology and subsequent negative follow-up biopsy. Group 2 patients had AUC and subsequent biopsy-confirmed lesions (5 HG UC, 8 LG UC, 2 atypia). Group 3 patients also had an initial diagnosis of AUC, but no evidence of UC or

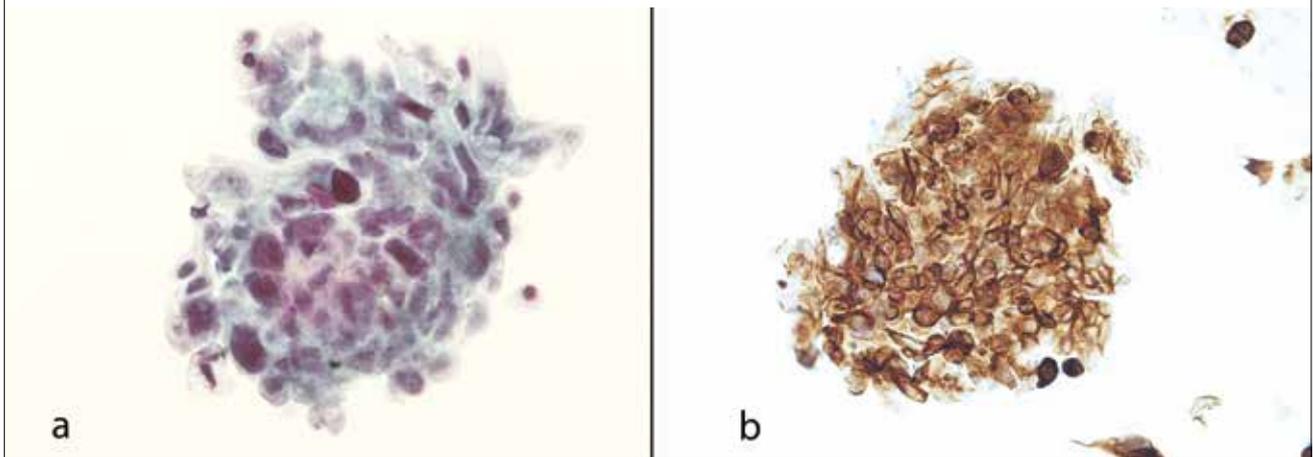
atypia at a subsequent biopsy.

Table III shows CK 20 results for all 50 urine cytological slides. Group 1 exhibited malignant cells with strong and positive antibody staining (Fig. 1). All benign cases were negative, except for a few cases in which umbrella cells were weak to moderately positive (Fig. 2). Of the 15 AUC in Group 2, 11 (73%) had atypical cells that stained strongly positive, (Figs. 3,4) while 4 (26.6%) were CK 20 negative. Of the 7 AUC in Group 3, in 6 (86%) AUC CK 20 staining was negative. The patient with a negative histological follow-up and CK 20 expression in the AUC smear had repeated atypical cytologies at follow-up and we cannot exclude this as a histological false negative.

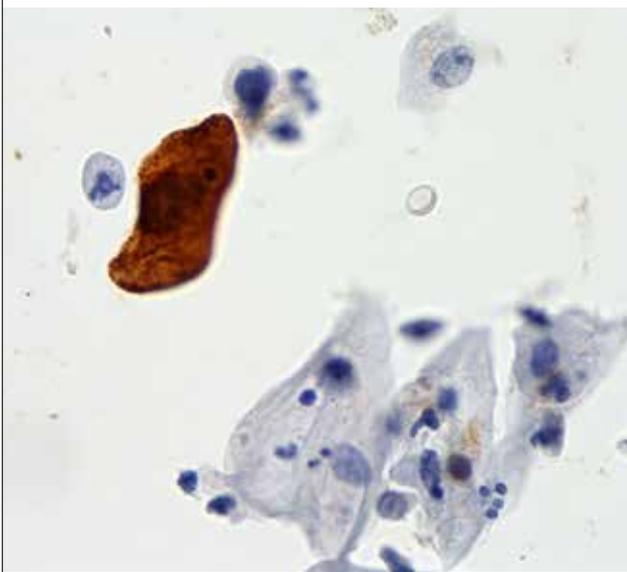
## Discussion

Although neoplastic urothelial cells were first recognized in the urine in 1864, it was not until the year 1945 that Papanicolaou and Marshall described the utility of urine cytology for the diagnosis of urothelial malignan-

**Fig. 1.** The same neoplastic cell clusters before and after CK 20 imuno-staining. a) Papanicolaou smear, 200x; b) CK 20 imuno-staining, 200x.



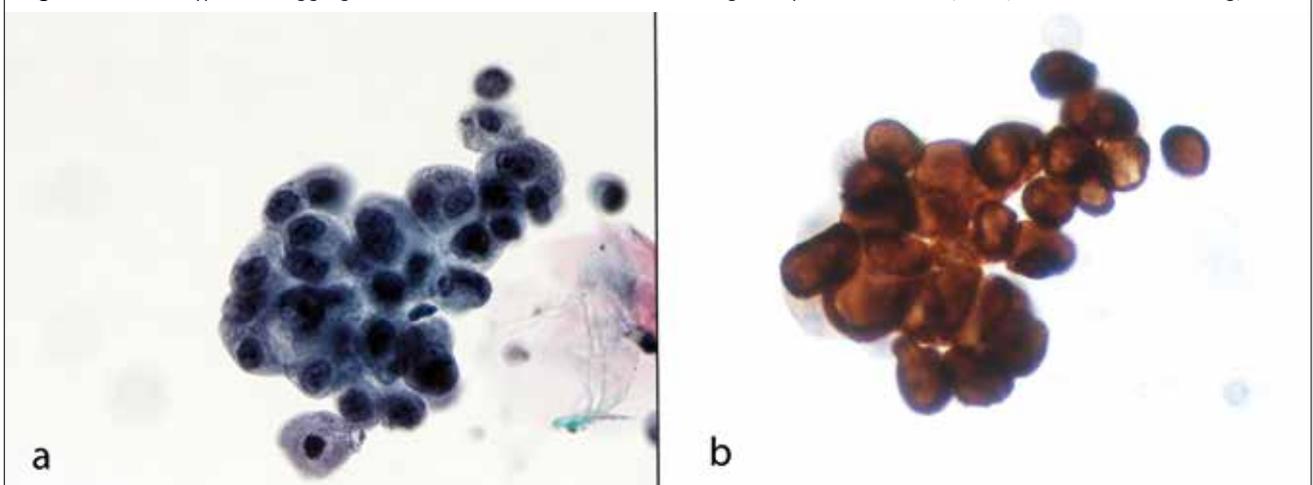
**Fig. 2.** Image of negative CK 20 staining cells and occasional superficial umbrella cell showing a positive staining pattern, 600x.



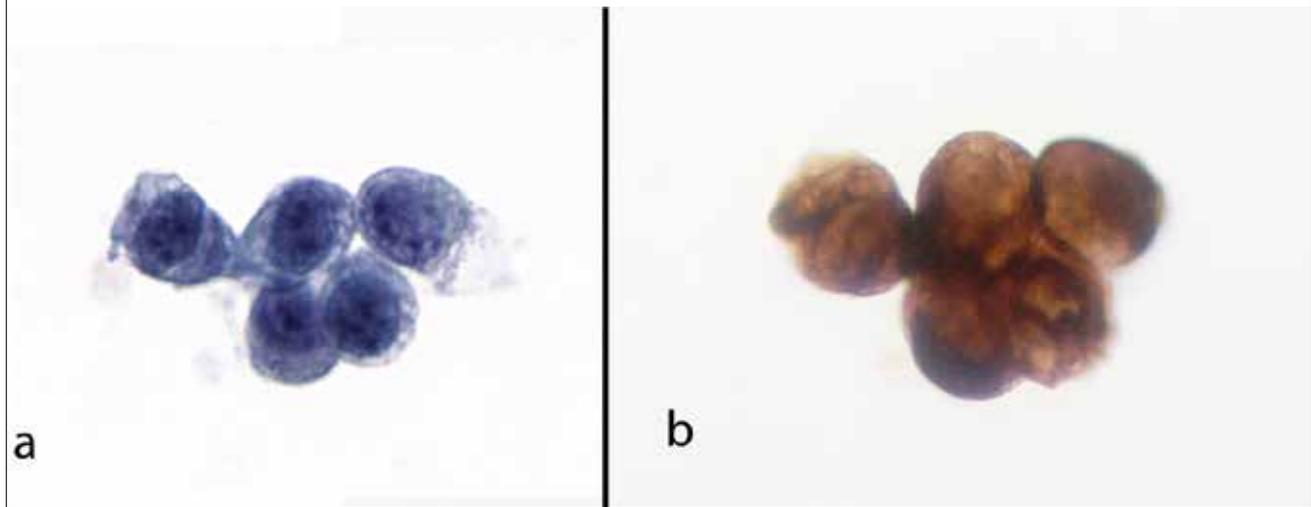
cy<sup>24</sup>. Since then, the effectiveness of primary detection or surveillance (using cytology combined with cytoscopy), and test performance metrics have been well-described in the literature. At our institute, urinary cytology combined with cytoscopy is a valuable tool for the diagnosis of urothelial neoplasia and its follow-up. To calculate sensitivity and specificity, we used histological diagnosis as a “gold standard”. The biopsy follow-up is generally accepted as the gold standard, even though this generates a bias due to the exclusion of patients without histological biopsy specimens and the inclusion of patients with false negative histological biopsy results. We collected and analyzed urine from three consecutive days on a single slide to obtain more representative samples, while limiting discomfort for the patient and load for the laboratory<sup>25</sup>.

In our cytohistological correlations, an atypical diagnosis was considered indicative of the neoplastic process. As previously found by Raab et al.<sup>7</sup>, this increases the sensitivity of urine cytology, albeit with a decrease in specificity. Our overall sensitivity was 65%, similar to

**Fig. 3.** The same atypical cell aggregate before and after CK 20 imuno-staining. a) Papanicolaou smear, 600x; b) CK 20 imuno-staining, 600x.



**Fig. 4.** The same atypical aggregate cell cluster before and after CK 20 imuno-staining. a) Papanicolaou smear, 600x; b) CK 20 imuno-staining, 600x.



the sensitivity reported by Bastacky et al.<sup>4</sup> (64%), and higher compared with data reported in reviews and meta-analysis. Our specificity was in the 95% confidence interval of the median specificity reported in the literature (0.83-0.997%)<sup>26</sup>. In an attempt to improve the cytological diagnosis of bladder malignancy, we focused on cytokeratin 20 as a marker. According to a recent review<sup>3</sup>, CK 20 sensitivity and specificity are 65-90% and 67-90% respectively, therefore comparable to other ancillary methods, e.g. fish (UroVysion) (30-86% sensitivity and 75-90% specificity) and uCyt (Immunocyt) (67-90% sensitivity and 62-84% specificity). However, this review also suggested that the immunostaining technique is simpler and easier to interpret than other tests, and it can aid in excluding benign condition to improve specificity. Umbrella cells can be easily distinguished from malignant and atypical cells (individual or aggregate cells) by their lack of atypia and architectural features, respectively. Therefore, CK 20-positive umbrella cells will not cause potential problems if the pathologist considers the CK 20 positivity of previously marked suspicious or atypical cells (e.g. from PAP smears). In regard to AUC, CK 20 immunocytochemical studies conducted on archived slides showed this marker is a potential adjunct for the triage of atypical urine cytology<sup>17-19 27</sup>.

In the present study, the positivity rate of CK 20 in cytologically diagnosed UC and AUC was 100% and 73.33%, respectively. This is in agreement with a previous CK 20 immunocytochemistry study by Li Min et al.<sup>27</sup>, who observed a CK 20 positivity rate of 96.8% and 63.6% in liquid-based cytologically diagnosed carcinoma and AUC, respectively. The difference in our CK 20 positivity rate between biopsy-positive and biopsy-negative AUC was significant ( $P < 0.05$ ). Also Lin et al., who tested CK 20 in a larger number of AUC cases in a previous and similar study, found that the difference in CK 20 positivity between biopsy-positive and biopsy-

negative AUC was significant ( $P < 0.001$ ). Based on our data, we conclude that the addition of CK 20 immunostaining to cytological evaluation improves diagnostic accuracy by confirming malignant diagnoses in suspicious cases, and correctly identifying the majority (73%) of “favor malignant” AUC on the basis of their strong cytoplasmic CK 20 staining (PPV = 91.6%), allowing urologists to make decisions and pathologists to improve their interpretation phase.

The small sample size was the major limitation of this study, especially with respect to benign biopsy AUC. Despite this, our results favorably compare with previous results in the literature for the accuracy of urinary cytology in daily practice<sup>4</sup> and establish the value of CK 20 immunocytochemistry as an adjuvant to urine cytology in the detection of UC and in assessing positivity in cases difficult to diagnose by morphology alone<sup>17 18</sup>. Nevertheless, studies with larger numbers of cases are necessary to confirm these results and verify CK 20 as a marker in urethral and retrograde catheterization specimens as well (where reactive and malignant criteria overlapping is more relevant<sup>19</sup>), and in follow-up for patients where malignant cells, fewer in number, are frequently obscured by inflammatory cells.

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