Keratin 17 Is a Novel Cytologic Biomarker for Urothelial Carcinoma Diagnosis

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ABSTRACT

Objectives: *The microscopic features of urine cytology specimens are subjective and may not reliably distinguish between benign urothelial cells and low-grade urothelial carcinoma (UC). Prior studies demonstrated that keratin 17 (K17) detection in biopsies is highly sensitive for UC. The current study aimed to define K17 diagnostic test performance for initial screening and detect recurrent UC in urine specimens.*

Methods: *K17 was detected by immunocytochemistry (ICC) in consecutively collected urine specimens (2018-2019). A qualitative score for the K17 test was determined in 81 samples (discovery cohort) and validated in 98 samples (validation cohort). K17 sensitivity and specificity were analyzed in both cohorts across all grades of UC.*

Results: *Based on the discovery cohort, the presence of 5 or more K17 immunoreactive urothelial cells (area under the curve = 0.90;* $P < .001$*) was the optimal threshold to define a K17-positive test. The sensitivity of the K17 ICC test for biopsy-confirmed UC was 35 of 36 (97%) and 18 of 21 (86%) in the discovery and validation cohorts, respectively. K17 was positive in 16 of 19 (84%) specimens with biopsy-confirmed low-grade UC and in 34 of 34 (100%) of specimens with high-grade UC.*

Conclusions: *K17 ICC is a highly sensitive diagnostic test for initial screening and detection of recurrence across all grades of UC.*

Key Points

- K17 ICC is more sensitive and specific than urine cytology across all grades of UC for initial screening and detecting recurrence.
- K17 ICC results, in contrast to cytologic classifications, are either positive or negative for UC, making integration into the clinical workflow straightforward.
- K17 is a highly sensitive and specific biomarker that can detect UC in atypical urine cytology.

Urothelial carcinoma (UC) is the most common cancer of the urinary tract, contributing to 4.7% of all cancer cases and resulting in significant morbidity and mortality in the United States.[1] Approximately 70% of UCs are detected at an early stage[2] and can be effectively treated if accurately diagnosed.[3-6] Although urine cytology is widely used to screen for UC, it has low sensitivity for the detection of early-stage, low-grade UC and is subject to inter- and intraobserver variability, leading to false-negative test results.[7] In addition, low-grade UCs have a high rate of recurrence and require frequent follow-up using cystoscopy with biopsy of suspicious lesions that may still fail to find small, in situ, or superficially invasive lesions.[8] Furthermore, although several FDA-approved biomarker-based urine tests for UC enhance diagnostic accuracy, they also have limited sensitivity for low-grade UCs.[9-17] Thus, there is an unmet clinical need to identify a highly sensitive and specific biomarker for initial screening and to monitor for recurrence of UC across all grades to enable timely treatment of UC.

Keratin 17 (K17) is typically expressed during embryonic development, silenced in most adult somatic tissues, and reexpressed in a range of cancer types.[18] We initially discovered K17 as a biomarker of aggressive cervical cancer through an unbiased proteomic screen[19] and subsequently showed that K17 expression was a negative prognostic biomarker in a range of other cancer types.[20-26] To assess its potential role as a biomarker in UC, we evaluated K17 expression in tissue specimens by RNA sequencing data mining and confirmed by immunohistochemistry. We used urothelial biopsies for discovery and validation and found that K17 was expressed in 100% of UCs but not in normal urothelial mucosa.[27,28] Furthermore, we performed K17 immunocytochemistry (ICC) on a pilot set of urine specimens with a defined cytologic diagnosis and found that K17 ICC was 100% sensitive and 96% specific as a cytologic biomarker for UC.[28] Based on these findings, we hypothesized that K17 ICC could be used as a noninvasive, highly sensitive and specific diagnostic urine cytologic biomarker for all grades of UC for both initial screening and subsequent screening for recurrence.

Materials and Methods

Case Selection

A total of 179 remnant ThinPrep CytoLyt (Hologic)–fixed urine specimens were collected between 2018 and 2019 from participants 18 years of age or older with hematuria (blood in urine) or followed to detect recurrence of UC following treatment (Supplementary Figure 1; all supplemental materials can be found at *American Journal of Clinical Pathology* online). Clinicopathologic information included concurrent grade from corresponding biopsy diagnoses, age, sex, smoking history, and history of UC or other cancer. Patient confidentiality was protected per an institutional review board–approved protocol (CORIHS 94651). Of the 179 consecutive collected samples, 81 samples collected from the first 4 months (December 2018-March 2019) were used to determine standardized quantitative scoring criteria to define a K17 ICC–positive test (discovery cohort). Based on the power analysis using the K17 ICC sensitivity and specificity from the discovery cohort, we prospectively collected 98 samples (validation cohort) over the next 4 months (April-August 2019) to evaluate the sensitivity and specificity of K17 ICC using the quantitative scoring criteria determined from the discovery cohort.

K17 Immunocytochemistry

Urine samples were centrifuged at 1000*g* for 10 minutes; the pelleted cells were resuspended in 20 mL of PreservCyt (Hologic) and transferred to charged-glass slides using a ThinPrep 2000 processor. The slides were stained using Autostainer Link 48 (Agilent Technologies). Endogenous peroxidase activity was blocked using EnVision FLEX wash peroxidase-blocking reagent (Agilent Technologies). Following incubation with anti-K17 antibody (KDX K17; 1:5,000 dilution), slides were processed by a direct polymer-based immunoperoxidase method using EnVision FLEX HRP, developed in EnVision FLEX DAB+ chromogen, and counterstained with hematoxylin. Slides were dehydrated in graded ethanols and cover slipped.

Slides were screened by a cytotechnologist to count the total number of K17-positive urothelial cells per slide. K17 slides were independently scored as positive or negative by each of the 2 participating pathologists, who were blinded to the urine cytology or biopsy diagnosis.

Statistical Analysis

K17 staining in urothelial cells was evaluated based on subjective assessment by a pathologist as absent (0), light (±1), or strong (±2) (Supplementary Figure 2). Only urothelial cells with 2± intensity K17 staining were counted to arrive at a final score for each slide. Mann-Whitney and receiver operating characteristic (ROC) curve analyses were used to determine standardized qualitative scoring criteria to define K17 ICC–positive test results. The minimum threshold for the number of strongly K17-positive cells, which provided optimal sensitivity and specificity (using the biopsy diagnosis as the gold standard) was determined in the discovery cohort; this threshold was subsequently evaluated in the validation cohort. Kappa statistics were used to calculate the interreader reproducibility between the 2 pathologists. The sample sensitivity and specificity of K17 ICC for the detection of biopsy-confirmed UC was calculated by comparison with a concurrent or prior histologic diagnosis of UC. Samples that had no history of abnormal urine cytology or tissue diagnosis of carcinoma were categorized as negative for UC. Statistical significance was set at $P < .05$, and analyses were performed using SAS statistical software, version 9.4 (SAS Institute) and Prism statistical software, version 7 (GraphPad Software).

Results

K17 Expression by ICC in 5 or More Strong Positive Urothelial Cells Defines a Positive Test in Urine Specimens

In our prior pilot study,[28] a K17 ICC–positive test was based on the presence of staining in urothelial cells,

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irrespective of the number of stained cells. The current study sought to refine the assay through the determination of a precise quantitative threshold for the classification of positive test results and to evaluate test performance in an independent validation cohort. Immunocytochemically stained slides from the discovery cohort, totaling 81 specimens, were screened for K17 staining in urothelial cells Table 1. K17 staining was not seen in benign urothelial cells Figure 1A but faint cytoplasmic staining was occasionally seen in benign squamous epithelial cells, most commonly in urine specimens from female patients Figure 1B. Strong staining was detected in urothelial cells Figure 1C and the total number of strongly stained cells per slide ranged from 0 to more than 100 cells. The optimal threshold to define positive test results in urine cytology relative to the presence or absence of UC on corresponding tissue biopsy was 5 or more strongly stained K17 urothelial cells (ROC area under the curve = 0.90; $P < .001$) Figure 1D. Based on this threshold, 2 cytopathologists (K.R.S. and M.W.) agreed on the scoring of K17 ICC test results in 88% of cases, with a κ value of 72% (95% CI, 53%-93%). Any discordance in the cases between 2 cytopathologists was resolved by recounting the number of positive urothelial cells.

The sensitivity of the K17 ICC test in the discovery cohort was 35 of 36 (97%; 95% CI, 86%-100%), and the specificity was 39 of 45 (86%; 95% CI, 74%-94%) for UC Table 2. We subsequently validated the K17 test threshold in a second set of prospectively collected urine samples (n = 98) and found that K17 ICC had a sensitivity of 18 of 21 (86%; 95% CI, 65%-95%) and specificity of 71 of 77 (92%; 95% CI, 84%-96%) for UC (Table 2).

Four cases that initially appeared to have false-negative test results by K17 ICC from both cohorts were later found to have K17-positive low-grade UCs on the consecutive biopsies. Twelve urine specimens with negative cytologic findings were positive for K17 but had no current or prior biopsy diagnosis of UC and were deemed to be false-positive test results. Thus, K17 ICC was sensitive for UC based on the detection of 5 or more strongly stained urothelial cells in both the discovery and the validation cohorts.

K17 ICC Is Highly Sensitive and Specific Across All Grades of UC

Of the 179 patients comprising the combined discovery and validation cohorts, 76 of 179 (42%) cases had

Table 1
Patient and Tumor Characteristics

	Total (n = 179)	Discovery Cohort[a] (n = 81)	Validation Cohort[b] (n = 98)
Age at diagnosis, mean ± SD, y	68.58 ± 15.49	69.01 ± 16.50	68.26 ± 15.11
Sex, No. (%)			
Female	99 (55)	40 (49)	59 (60)
Male	80 (45)	41 (51)	39 (40)
Clinical indication, No. (%)			
Screening with hematuria	76 (42)	40 (49)	36 (37)
Recurrence follow-up (with or without hematuria)	62 (35)	37 (46)	25 (26)
Others[c]	41 (23)	4 (5)	37 (38)
Urine cytology diagnosis, No. (%)			
Negative for malignancy	114 (64)	33 (41)	81 (83)
Mild atypia	41 (23)	32 (40)	9 (9)
Moderate atypia	10 (6)	9 (11)	1 (1)
Severe atypia/suspicious for malignancy	5 (3)	2 (2)	3 (3)
Positive for malignancy	9 (5)	5 (6)	4 (4)
Smoking status[d], No. (%)			
Nonsmoker	12 (7)	4 (5)	8 (8)
Current smoker	11 (6)	5 (6)	6 (6)
Previous smoker	34 (19)	23 (28)	11 (11)
Urothelial biopsy diagnosis, No. (%)			
PUNLMP	5 (3)	2 (2)	3 (3)
Carcinoma in situ	10 (6)	5 (6)	5 (5)
Noninvasive LG PUC	19 (11)	8 (10)	11 (11)
Noninvasive HG PUC	13 (7)	9 (11)	4 (4)
Invasive UC	11 (6)	7 (9)	4 (4)
Follow-up time, mo	18	11	7

HG, high grade; ICC, immunocytochemistry; K17, keratin 17; LG, low grade; PUC, papillary urothelial carcinoma; PUNLMP, papillary urothelial neoplasm of low malignant potential; SD, standard deviation; UC, urothelial carcinoma.
[a]Prospectively collected samples used to determine a standardized quantitative scoring threshold to define K17 ICC–positive test.
[b]Second prospectively collected samples used to validate evaluate K17 test performance using the quantitative scoring threshold determined from the discovery cohort.
[c]Others included cystitis, urinary retention, hydronephrosis, other specified disorders of the bladder, mixed incontinence, urinary tract infection, and renal calculi.
[d]Smoking status was available for 57 cases only.

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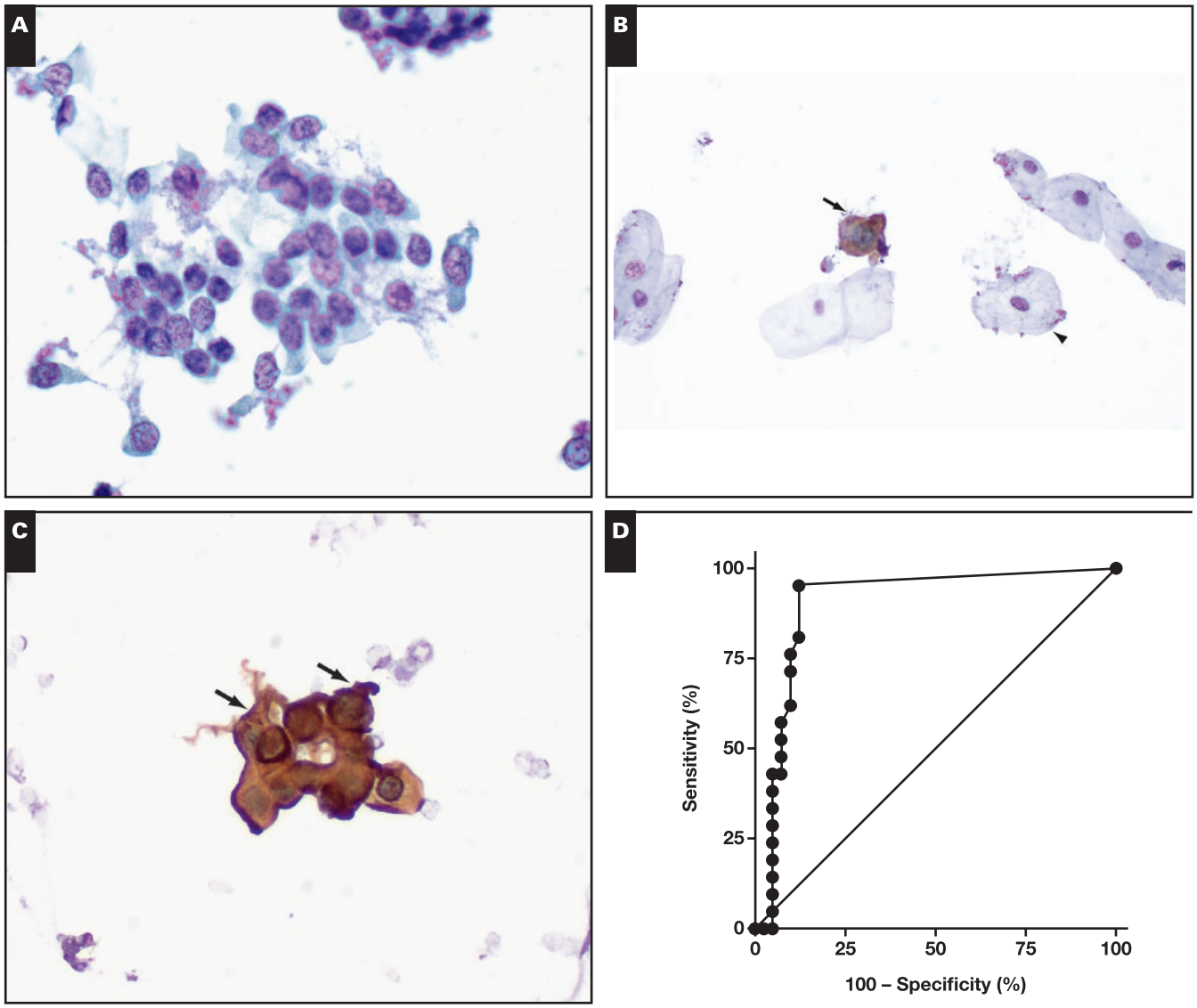

Figure 1 Detection of Keratin 17 in 5 or more strong positive urothelial cells defines a positive test. **A**, Benign urothelial cells. **B**, Keratin 17 (K17)–positive urothelial cell (arrow) adjacent to benign squamous cell (arrowhead). **C**, Urothelial cells (arrows) showing strong K17 staining. **D**, The optimal cutoff value from receiver operating characteristic (ROC) curves was determined using the Youden index (area under the curve = 0.9013; $P < .0001$). The optimal cutoff value in the resulting ROC curve corresponded to 5 or more positive urothelial cells for determining a K17 immunocytochemistry (ICC)–positive test (**C**) or K17 ICC–negative test (**A** and **B**).

Table 2

Keratin 17 Immunocytochemistry Is a Highly Sensitive Diagnostic Test for Urothelial Carcinoma Based on the Established Quantitative Score in the Discovery and Validation Cohorts

	Discovery K17 ICC, No. (%; 95% CI)	Validation K17 ICC, No. (%; 95% CI)
Sensitivity	35/36 (97; 86-100)	18/21 (86; 65-95)
Specificity	39/45 (86; 74-94)	71/77 (92; 84-96)

CI, confidence interval; ICC, immunocytochemistry; K17, keratin 17.

only a history of hematuria (screened for UC), 62 of 179 (35%) cases were followed for UC recurrence after treatment, and the remaining 41 of 179 (23%) cases had a history of urinary tract infection, kidney stones, cystitis, urinary retention, hydronephrosis, "mixed incontinence," or other specified disorders of the bladder. These cases were grouped together as "other category" and were not included for the stratified analysis.

Among the cases with a history of hematuria (screened for UC) or monitored for recurrence with

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biopsy-confirmed UC, the K17 test was positive in 16 of 19 (84%) with low-grade UC and 34 of 34 (100%) with high-grade UC Table 3. By contrast, urine cytology was classified as suspicious or positive for UC in 3 of 19 (16%) of low-grade and 3 of 34 (9%) of high-grade UCs, suggesting that urine cytology was less sensitive than K17 ICC.

K17 ICC was also positive in 30 of 52 (58%) urine specimens that had a cytologic diagnosis of atypia. Among these cases, the sensitivity of K17 ICC was 27 of 29 (93%; 95% CI, 78%-99%), and the specificity was 20 of 23 (87%; 95% CI, 68%-95%), with a positive predictive value (PPV) of 27 of 30 (90%; 95% Cl, 74%-97%) and a negative predictive value (NPV) of 20 of 22 (91%; 95% Cl, 72%-98%) for the subsequent biopsy diagnosis of UC Table 4. Thus, K17 ICC detected the underlying UC with high sensitivity in atypical urine cytology specimens.

K17 ICC Is Sensitive and Specific for Both Initial Screening and Recurrence Testing Across All Grades of UC

To evaluate the sensitivity and specificity of K17 ICC for initial screening in patients with hematuria and to detect UC recurrence following treatment in patients with or without hematuria, we categorized cases based on their status at the time of diagnosis, including initial screening (n = 76) or follow-up for recurrence (n = 62). K17 ICC was positive in 22 of 76 (29%) of specimens with hematuria, with a sensitivity of 11 of 11 (100%; 95% CI, 74%-100%), a specificity of 54 of 65 (83%; 95% CI, 72%-90%), a PPV of 11 of 22 (50%; 95% Cl, 31%-70%), and an NPV of 54 of 54 (100%; 95% Cl, 93%-100%) for UC Table 5. K17-positive test results included 11 samples that had biopsy-confirmed UC but were negative by urine cytology. K17 ICC was also positive in 11 urine specimens with negative cytology that had no current or prior biopsy diagnosis of UC and thus were concluded to be false-positive K17 ICC test results. By contrast, urine cytology of samples with hematuria had a sensitivity of 0 of 11 (0%), a specificity of 65 of 65 (100%; 95% CI, 94%-100%), a PPV of 0, and an NPV of 65 of 76 (86%; 95% CI, 6%-92%) (Table 5).

K17 ICC was positive in 40 of 62 (65%) of specimens submitted to screen for UC recurrence, with a sensitivity 40 of 44 (92%; 95% CI, 78%-96%), a specificity of 18 of 18 (100%; 95% CI, 82%-100%), a PPV of 40 of 40 (100%; 95% Cl, 91%-100%), and an NPV of 18 of 22 (82%; 95% Cl, 61%-93%) Table 6. By contrast, urine cytology for samples submitted to test for recurrent UC had a sensitivity of 10 of 44 (23%; 95% CI, 13%-37%), a specificity of 15 of 18 (83%; 95% CI, 61%-94%), a PPV of 10 of 13 (77%; 95% Cl, 50%-92%), and an NPV of 15 of 49 (31%; 95% Cl, 20%-45%) (Table 6). The 4 K17 ICC samples initially deemed to be false negatives were later confirmed to have K17-positive low-grade UC on a subsequent biopsy.

Thus, K17 ICC was sensitive and specific to detect UC for initial screening and to monitor for recurrence across all grades of UC. In addition, 2 cases initially deemed positive for K17 ICC but negative by cystoscopy were later confirmed to have UC of the renal pelvis,

Table 3

Keratin 17 Immunocytochemistry Is More Sensitive Than Cytology for Low-Grade and High-Grade Urothelial Carcinoma, Combined Discovery, and Validation Cohorts[a]

Diagnosis	Samples, No.	Cytology Sensitivity, No. (%; 95% CI)	K17 ICC Sensitivity, No. (%; 95% CI)
Low-grade UC[b]	19	3/19 (16; 6-37)	16/19 (84; 62-94)
High-grade UC[c]	34	3/34 (9; 2-29)	34/34 (100; 83-100)

CI, confidence interval; ICC, immunocytochemistry; K17, keratin 17; UC, urothelial carcinoma.
[a]Analysis is based only on the 53 cases with biopsy-confirmed low- or high-grade UC.
[b]Includes noninvasive, low-grade papillary carcinoma.
[c]Includes noninvasive, high-grade papillary UC; invasive UC; and carcinoma in situ.

Table 4

Keratin 17 Immunocytochemistry in Atypical Urine Cytology Specimens

Test	K17 ICC, No. (%; 95% CI)
Sensitivity[a]	27/29 (93; 78-99)
Specificity[a]	20/23 (87; 68-95)
PPV	27/30 (90; 74-97)
NPV	20/22 (91; 72-98)

CI, confidence interval; ICC, immunocytochemistry; K17, keratin 17; NPV, negative predictive value; PPV, positive predictive value; UC, urothelial carcinoma.
[a]Sensitivity and specificity were calculated based on biopsy confirmation in 52 samples prospectively collected from Stony Brook Medicine for UC.

Table 5

Keratin 17 Immunocytochemistry Is More Accurate Than Cytology for Detecting Urothelial Carcinoma in Patients With Hematuria

Test	Cytology[a], No. (%; 95% CI)	K17 ICC, No. (%; 95% CI)
Sensitivity[b]	0/11	11/11 (100; 74-100)
Specificity[b]	65/65 (100; 94-00)	54/65 (83; 72-90)
PPV	0/0	11/22 (50; 31-70)
NPV	65/76 (86; 6-92)	54/54 (100; 93-100)

CI, confidence interval; ICC, immunocytochemistry; K17, keratin 17; NPV, negative predictive value; PPV, positive predictive value; UC, urothelial carcinoma.
[a]Negative cytology test results include samples with a diagnosis of no evidence of malignancy, reactive changes, acute inflammation, mild atypia, or moderate atypia.
[b]Sensitivity and specificity were calculated based on biopsy confirmation in 76 samples prospectively collected from Stony Brook Medicine for initial UC screening.

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Table 6

Keratin 17 Immunocytochemistry Is More Accurate Than Cytology for Detecting Recurrence of Urothelial Carcinoma

Test	Cytology[a], No. (%; 95% CI)	K17 ICC, No. (%; 95% CI)
Sensitivity[b]	10/44 (23;13-37)	40/44 (91; 78-96)
Specificity[b]	15/18 (83; 61-94)	18/18 (100; 82-100)
PPV	10/13 (77; 50-92)	40/40 (100; 91-100)
NPV	15/49 (31; 20-45)	18/22 (82; 61-93)

CI, confidence interval; ICC, immunocytochemistry; K17, keratin 17; NPV, negative predictive value; PPV, positive predictive value; UC, urothelial carcinoma.
[a]Negative cytology test results include samples with a diagnosis of no evidence of malignancy, reactive changes, acute inflammation, mild atypia, or moderate atypia. Positive urine cytology test results included samples scored as severe atypia/suspicious for carcinoma or positive for carcinoma. Four false-negative samples by K17 ICC were later confirmed to have K17-positive low-grade UCs on consecutive bladder biopsies.
[b]Sensitivity and specificity were calculated based on biopsy confirmation in 62 prospectively collected samples from Stony Brook Medicine for UC recurrence follow-up.

suggesting that K17 ICC may also have a role in testing for UC of the upper urinary tract.

Discussion

Currently available cytology assays do not reliably distinguish between low-grade UCs and benign lesions. Based on our finding that K17 is a highly sensitive and specific biomarker that can detect both low- and high-grade UC in urothelial tissue, we set out to determine K17 ICC test performance in urine specimens and found that K17 ICC of urine specimens had a sensitivity of 100% as an initial screening test for hematuria and 91% for the detection of recurrent UC across all grades of UC. Thus, K17 ICC has the potential to increase diagnostic accuracy for UC and could be used to triage the patients most likely to benefit from therapeutic intervention while obviating the need for cystoscopy for patients with K17-negative test results. In addition, K17 ICC test results, in contrast to cytologic classifications, are dichotomous—either positive or negative for UC—making integration into the clinical workflow straightforward. Furthermore, our data support the conclusion that K17 ICC could be used for triage of patients with atypical urine cytology cases. Together, these results suggest that K17 ICC is more sensitive and specific than urine cytology across all grades of UC for initial screening and to detect recurrence.

Urine cytology is widely used to screen for UC but has low sensitivity for the detection of early-stage, low-grade UC, leading to false-negative test results. Recently, The Paris System (TPS) of urine cytology reporting was proposed to reduce the number of atypical diagnoses and increase the accuracy for reporting high-grade UC using urine cytology. The TPS diagnostic categories include the clinically actionable categories of atypia (class III), low-grade UC (class V), and high-grade UC (class VI). By contrast, cases that are "suspicious" for high-grade UC are defined as class IV, but there is no corresponding diagnostic class for cases that could be considered "suspicious for low-grade UC."[29-33] This gap in the diagnostic category reflects the difficulty in the cytologic diagnosis of low-grade UCs that can be indistinguishable from benign specimens that result from instrumentation, lithiasis, or other processes that have no premalignant potential. Although the TPS criteria have improved the sensitivity for detection of high-grade UC, they fail to provide criteria to differentiate between benign lesions and low-grade UC, potentially missing opportunities for early diagnosis and treatment of low-grade UC, the most prevalent neoplasms that urologists encounter. Because K17 expression is specific to malignant cells, the K17 ICC may enable the detection of low-grade UC in cases with equivocal "suspicious" findings that TPS does not address.

One limitation of the current study is that we calculated K17 ICC sensitivity based on the concurrent biopsy diagnosis of UC. Cases were considered false positive when K17 ICC was positive but no concurrent biopsy diagnosis was available. To assess the performance of K17 ICC more definitively, a prospective clinical trial in which all patients with positive K17 ICC test results are followed up with a concurrent gold-standard biopsy would be required.

To improve the diagnostic accuracy for UC in urine specimens, several commercial tests have been developed,[8,34-37] including the UroVysion Kit (Abbott Molecular), nuclear matrix protein 22 (NMP22), bladder tumor antigen (BTA), ImmunoCyt (DiagnoCure), and Cxbladder (Pacific Edge), which are FDA approved to detect UC recurrence in combination with urine cytology and cystoscopy.[11,13,14,37-40] Although some of these tests provide enhanced diagnostic sensitivity, there are limitations to their implementation in clinical practice. UroVysion focuses on the detection of aneuploidy, which may be negative in low-grade UC,[38,41,42] and NMP22 and BTA focus on the detection of tumor-associated antigens and may show false-positive test results in the absence of UC.[11] Indeed, most of these tests provide enhanced sensitivity but fail to demonstrate better specificity compared with urine cytology, and none of these tests have the ability to accurately detect low-grade UC or replace urine cytology. By contrast, K17 ICC is sensitive and specific for both low-grade and high-grade UC. Thus, if clinically implemented, K17 ICC could be used to identify patients who are unlikely to require treatment while focusing clinical resources on the patients most likely to benefit from

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cystoscopy and biopsy, facilitating early therapeutic intervention and improving clinical outcomes.

K17 ICC is a sensitive and specific cytologic test for primary screening of samples with hematuria and to monitor for disease recurrence across all grades of UC. Thus, the K17 ICC could serve as a diagnostic adjunct to guide the clinical management of patients with UC. These observations provide evidence to support the development of prospective clinical trials to further define the clinical and diagnostic impact of K17 ICC.

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