



Original Investigation | Urology

Urine Methylation Test for Urothelial Cancer Diagnosis and Surveillance

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Abstract

IMPORTANCE Urothelial carcinoma (UC) is characterized by frequent recurrence, and accurate noninvasive tests remain needed for early detection and postoperative surveillance.

OBJECTIVE To develop and validate a urine-based assay targeting *ALO21918.2* and vimentin (*VIM*) methylation for UC detection and postoperative surveillance.

DESIGN, SETTING, AND PARTICIPANTS This prospective, blinded, multicenter diagnostic study was performed at 3 tertiary medical centers in China. Samples were collected between December 1, 2021, and June 25, 2025. Consecutively enrolled adults with UC, benign urinary diseases, or nonurothelial malignant disease were included in a single-center training cohort, a 3-center validation cohort, and a prospective surveillance cohort. The surveillance cohort had a median follow-up of 6 months, completed December 13, 2023.

EXPOSURE Urinary DNA methylation was assessed using quantitative methylation-specific polymerase chain reaction (PCR) analysis.

MAIN OUTCOMES AND MEASURES The primary outcomes were the sensitivity and specificity of the methylation assay for UC detection. Secondary outcomes included diagnostic performance according to tumor stage and grade and comparisons with urine cytology and fluorescence in situ hybridization (FISH).

RESULTS This analysis included a total of 2307 participants, consisting of 643 in the training cohort (median age, 64 [IQR, 56-71] years; 494 men [76.8%]), 1620 in the validation cohort (median age, 67 [IQR, 61-73] years; 1129 men [69.7%]), and 44 with UC in the surveillance cohort (median age, 62 [IQR, 59-72] years; 32 men [72.7%]). The assay demonstrated sensitivities of 91.2% (95% CI, 87.6%-93.9%) in the training cohort and 90.8% (95% CI, 88.4%-92.8%) in the validation cohort and specificities of 90.0% (95% CI, 85.9%-93.2%) in the training cohort and 93.2% (95% CI, 91.3%-94.7%) in the validation cohort. In the training and validation cohorts, sensitivity remained high for Ta UC (79.6% [95% CI, 64.3%-89.7%] and 84.3% [95% CI, 77.8%-89.2%], respectively) and low-grade UC (86.4% [95% CI, 77.0%-92.5%] and 82.5% [95% CI, 75.7%-87.8%], respectively). Compared with urine cytology and FISH, the methylation assay demonstrated higher sensitivity while maintaining comparable specificity. After surgery, urinary methylation results were negative in 30 of 32 patients (93.8%) with positive preoperative results. During surveillance, the assay detected all 9 recurrences with a median lead time of 4.5 (IQR, 1.5-5.5) months before clinical confirmation and remained negative in 29 of 35 patients (82.9%) without recurrence.

(continued)

Key Points

Question Can a noninvasive urine methylation assay accurately detect urothelial carcinoma and facilitate postoperative recurrence surveillance?

Findings In this diagnostic study including a training cohort of 643 participants and a multicenter validation cohort of 1620 participants, a noninvasive urine-based assay targeting *ALO21918.2* and *VIM* methylation achieved 90.8% sensitivity and 93.2% specificity for urothelial carcinoma detection. In a longitudinal surveillance cohort, the assay identified all 9 observed recurrences and provided a median lead time of 4.5 months before clinical confirmation.

Meaning This methylation assay may serve as a complementary noninvasive approach for urothelial carcinoma detection and postoperative recurrence surveillance.

+ Supplemental content

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Abstract (continued)

CONCLUSIONS AND RELEVANCE In this diagnostic study, a noninvasive PCR-based assay showed high diagnostic accuracy for UC detection and identified all observed recurrences during surveillance. The assay may serve as an adjunct for UC diagnosis and postoperative surveillance.

TRIAL REGISTRATION Chinese Clinical Trial Registry Identifier: [ChiCTR2300078302](#)

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Introduction

Urothelial carcinoma (UC) is a prevalent malignant neoplasm originating from the urothelial lining of the urinary tract, characterized by a high risk of recurrence.^{1,2} Approximately 50% of high-risk patients with UC experience recurrence within 5 years after surgery.^{1,2} Even for non-muscle-invasive UC, the progression risk within 5 years can vary from less than 1% to greater than 40%, depending on the risk category.³ UC primarily presents as bladder cancer, accounting for 90% to 95% of cases. The remaining 5% to 10% are classified as upper urinary tract urothelial carcinoma (UTUC) or proximal urethral cancer.⁴⁻⁶ Globally, bladder cancer affects 573 278 individuals and causes 212 536 deaths annually.⁷ Although UTUC is less common, it is frequently diagnosed at an invasive stage because of its nonspecific clinical presentation and the limited accessibility of the upper urinary tract.⁸ Consequently, there remains an unmet need for accurate, noninvasive biomarkers capable of detecting both bladder cancer and UTUC, particularly for early diagnosis and recurrence surveillance.

Histopathologic evaluation obtained by endoscopic procedures remains the reference standard for UC diagnosis; however, these procedures may cause discomfort, particularly among older patients.⁹ Several noninvasive diagnostic methods, including urine cytology and fluorescence in situ hybridization (FISH), have been incorporated into clinical practice.^{10,11} However, these methods have limitations. For example, although urine cytology has a high specificity of 97.9%, its sensitivity remains limited (34.5%), especially for low-grade UC.¹⁰ These limitations underscore the need for accurate and practical urine-based molecular assays.

DNA methylation is an important epigenetic modification involved in tumor initiation, progression, and recurrence.^{12,13} Recent studies have indicated that urinary DNA methylation signals may serve as biomarkers for UC diagnosis. For instance, one assay that analyzes 15 DNA methylation biomarkers (Bladder EpiCheck; Nucleix) has demonstrated a sensitivity of 68.2% and specificity of 88.0% for recurrence monitoring in patients with non-muscle-invasive bladder cancer.¹⁴ Furthermore, this assay demonstrated a higher diagnostic sensitivity for UTUC detection than urinary cytology (83% vs 59%).¹⁵

In a previous study,¹⁶ a novel UC methylation biomarker, designated *AL021918.2* (NCBI [AL021918](#)) (Ensembl ENSG00000280950.1) was identified, and a dual-target panel was developed by combining this biomarker with *VIM* (NCBI [7431](#)) methylation. Our objective in conducting this phase 2 study was to develop and optimize a urine test (IUrisure; Wuhan Ammunition Life-tech Co Ltd), which incorporates *AL021918.2* and *VIM*, to increase its clinical utility in a training cohort and validate its diagnostic accuracy in a multicenter validation cohort. In addition, we systematically evaluated the performance of the assay in diagnosing early-stage, low-grade, and minimal UC and compared its diagnostic accuracy with urine cytology and FISH. Finally, we evaluated its ability to detect recurrence in a longitudinal surveillance cohort.

Methods

Study Design and Participants

This diagnostic study was conducted in accordance with the Declaration of Helsinki¹⁷ and was approved by the ethics committees of all participating hospitals prior to commencement. All

participants signed informed consent forms. Eligible participants were adults 18 years or older with pathologically confirmed primary or recurrent UC (bladder cancer or UTUC), benign urinary diseases, or untreated nonurothelial malignant neoplasms. Detailed inclusion and exclusion criteria are provided in the eMethods in [Supplement 1](#). We followed the Standards for the Reporting of Diagnostic Accuracy Studies (STARD) reporting guideline.

Samples collected between December 1, 2021, and June 25, 2025, were allocated into 4 predefined cohorts. Cohort 1 consisted of tumor and adjacent normal tissues for marker evaluation. Cohort 2 (training cohort) included urine samples used for assay development and cutoff optimization. Cohort 3 (validation cohort) was a prospective multicenter cohort of patients enrolled from 3 hospitals before surgery. Cohort 4 (surveillance cohort) was a prospective longitudinal cohort in which postoperative urine samples were collected approximately every 3 months to evaluate recurrence monitoring, with follow-up completed on December 13, 2023. During follow-up, 44 patients with UC underwent testing with the assay both preoperatively and at least once postoperatively. Patients subsequently received routine postoperative surveillance, including cystoscopy and/or imaging examinations, according to institutional protocols.

Clinicians were blinded to assay results throughout the follow-up period, and assay findings were not used to guide surveillance scheduling or clinical decision-making. At the end of follow-up, the performance of the assay for recurrence monitoring was evaluated using a composite reference standard incorporating both pathologic and clinical diagnoses to determine clinical recurrence.

Sample Processing and Methylation-Specific Quantitative Methylation-Specific Polymerase Chain Reaction Detection

A morning urine sample was collected before or after surgery and preserved immediately. DNA was extracted from a 1-mL urine sample, followed by bisulfite conversion. Tissue DNA underwent identical bisulfite treatment after extraction from formalin-fixed paraffin-embedded specimens. Detailed laboratory procedures are provided in the eMethods in [Supplement 1](#).

The assay detects methylated templates by quantitative methylation-specific polymerase chain reaction (PCR) using specific primers and fluorescent probes for *ALO21918.2* and *VIM*; their sequences are shown in eTable 1 in [Supplement 1](#). The PCR procedures are provided in the eMethods in [Supplement 1](#), and quantitative methylation-specific PCR results were considered valid only if the *ACTB* (internal control) cycle threshold (Ct) value was 34 or less. To minimize bias, the laboratory personnel performing the assay were blinded to the participants' clinical characteristics and pathologic diagnoses, and the pathologists were blinded to the quantitative methylation-specific PCR results. The methylation levels of the 2 target genes were represented by the difference in Ct values between the target gene and *ACTB* (Δ Ct). The area under the receiver operating characteristic curve obtained with 10-fold cross-validation was utilized to evaluate the discriminative performance of *ALO21918.2* and *VIM* between UC and control samples in cohort 2. The initial cutoff values (Δ Ct) for the 2 genes were determined by maximizing the Youden index and were subsequently optimized to integer values. A sample was designated as methylation positive if 1 or 2 target genes were methylated, based on the optimized cutoff values.

Analytic Performance Evaluation

The analytic performance of the assay was evaluated by assessing the limit of detection (LOD) and assay precision. Reference DNA templates were validated for methylation status by Sanger sequencing and for absolute concentration by droplet digital PCR.

Four methylation profiles of the 2 target genes were prepared for LOD testing: *VIM*-positive plus *ALO21918.2* positive, *VIM* negative plus *ALO21918.2* positive, *VIM* positive plus *ALO21918.2* negative, and *VIM* negative plus *ALO21918.2* negative. Each profile contained 3 DNA concentrations with matched stepwise methylation ratios (1%, 10%, and 50% for 100 copies/ μ L; 0.1%, 1%, and 5% for 1000 copies/ μ L; and 0.01%, 0.1%, and 0.5% for 10 000 copies/ μ L).

In total, 36 distinct reference templates were generated (4 methylation profiles × 3 DNA concentrations × 3 methylation ratios). Each template was tested across 3 independent runs with 20 technical replicates per run. LOD was defined as the minimal template concentration yielding 95% or greater positive amplification.

Assay reproducibility was evaluated using 6 reference panels (R1-R6) covering all 4 methylation status combinations: *VIM* negative plus *ALO21918.2* negative (R1), *VIM* negative plus *ALO21918.2* positive (R5), *VIM* positive plus *ALO21918.2* negative (R4), and *VIM* positive plus *ALO21918.2* positive (R2, R3, and R6). Intrabatch, interbatch, interday, and interoperator precision were assessed by testing each sample 20 times across 3 reagent batches on 20 consecutive days with 2 technicians. Precision was acceptable when the coefficient of variation of both targets was 5% or less.

Statistical Analysis

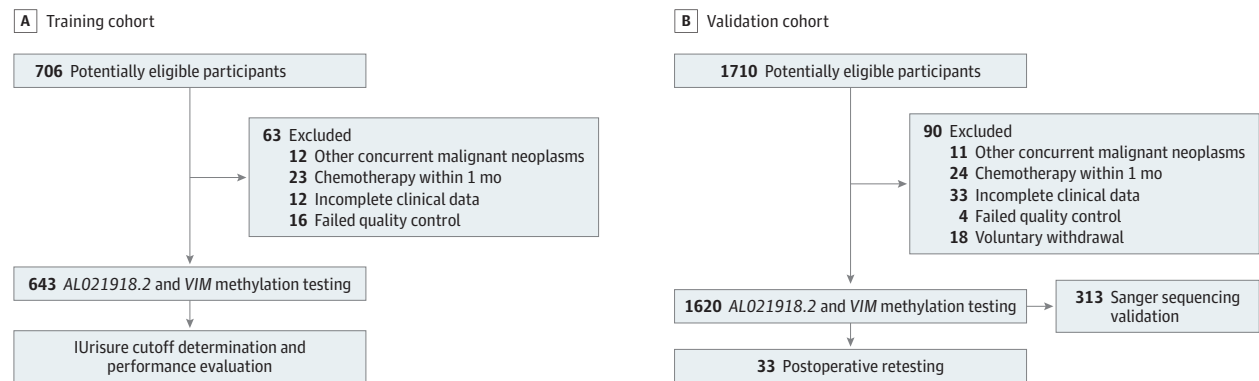
All statistical analyses were performed using R software, version 4.0.5 (R Project for Statistical Computing). Descriptive summaries of baseline demographic and clinical features were generated, with categorical variables expressed in proportions and continuous variables summarized using median and IQR. Because methylation levels were not normally distributed, comparisons between groups were performed using the Mann-Whitney test. A comparison of sensitivity and specificity between groups was performed using the χ^2 test or Fisher exact test. Differences in methylation levels of paired samples were analyzed using the Wilcoxon signed-rank test. Significance testing was 2 sided, and a threshold of $P < .05$ was used to indicate statistical significance.

Results

Baseline Characteristics

This study included 1 tissue sample dataset (cohort 1) and 3 urine sample datasets (cohorts 2, 3, and 4 [n = 2307]). Cohort 1 consisted of 164 UC and 28 healthy adjacent tissue samples for biomarker evaluation. Cohort 2 included 643 participants (median age, 64 [IQR, 56-71] years; 149 [23.2%] female and 494 [76.8%] male), and cohort 3 included 1620 participants (median age, 67 [IQR, 61-73] years; 491 [30.3%] female and 1129 [69.7%] male) (Figure 1). Cohort 4, a prospective, longitudinal follow-up cohort, included 44 patients with UC (median age, 62 [IQR, 59-72] years; 12 [27.3%] female and 32 [72.7%] male) who provided urine samples at multiple time points preoperatively and postoperatively (eFigure 1 in Supplement 1). Baseline characteristics of the 3 urine cohorts are detailed in Table 1, with cohort-specific characteristics provided in eTables 2 and 3 in Supplement 1.

Figure 1. Flowchart of the Training and Validation Cohorts



Analytic Robustness and Technical Feasibility

The methylation assay demonstrated high analytic reproducibility, with a coefficient of variation for both *ALO21918.2* and *VIM* below the prespecified 5% threshold across reagent batches, operators, and testing days (eFigure 2 in Supplement 1). The assay detected methylated target molecules at an absolute number of 10 copies at a background concentration of 1000 copies/μL (eTable 4 in Supplement 1). Among 313 samples evaluated by Sanger sequencing, the *ALO21918.2* methylation assay showed 99.4% concordance with sequencing results (95% CI, 97.7%-99.8%; κ = 0.99 [95% CI, 0.97-1.00]). The *VIM* methylation assay exhibited 99.0% concordance (95% CI, 97.2-99.7; κ = 0.98 [95% CI, 0.96-1.00]) (eTable 5 in Supplement 1).

Diagnostic Performance

Methylation levels of *ALO21918.2* and *VIM* were higher in samples with UC than in controls across both tissue and urine cohorts and increased progressively with advancing tumor stage and grade (eFigure 3 in Supplement 1). In cohort 2, optimal cutoff values for *ALO21918.2* and *VIM* genes were set

Table 1. Demographic and Pathologic Information of the 3 Urine-Based Cohorts

Group	Patient cohort, No./total No. (%)		
	Training (n = 643)	Validation (n = 1620)	Surveillance (n = 44)
Age, median (IQR), y	64 (56-71)	67 (61-73)	62 (59-72)
Sex			
Female	149/643 (23.2)	491/1620 (30.3)	12/44 (27.3)
Male	494/643 (76.8)	1129/1620 (69.7)	32/44 (72.7)
Overall UC	352/643 (54.7)	707/1620 (43.6)	44/44 (100)
Tumor type			
Primary	274/352 (77.8)	645/707 (91.2)	30/44 (68.2)
Recurrent	78/352 (22.2)	62/707 (8.8)	14/44 (31.8)
Tumor location			
Bladder	272/352 (77.3)	486/707 (68.7)	33/44 (75.0)
Ureteral	27/352 (7.7)	100/707 (14.1)	5/44 (11.4)
Renal pelvis	43/352 (12.2)	94/707 (13.3)	6/44 (13.6)
Urethral	NA	2/707 (0.3)	NA
Concurrent	10/352 (2.8)	25/707 (3.5)	NA
Tumor grade			
PUNLMP	12/352 (3.4)	19/707 (2.7)	NA
Low grade	88/352 (25.0)	166/707 (23.5)	5/44 (11.4)
High grade	241/352 (68.5)	513/707 (72.6)	39/44 (88.6)
Unknown	11/352 (3.1)	9/707 (1.3)	NA
Tumor category			
Tis	8/352 (2.3)	9/707 (1.3)	3/44 (6.8)
Ta	44/352 (12.5)	172/707 (24.3)	5/44 (11.4)
T1	190/352 (54.0)	260/707 (36.8)	31/44 (70.5)
T2	60/352 (17.0)	86/707 (12.2)	3/44 (6.8)
T3	25/352 (7.1)	69/707 (9.8)	2/44 (4.5)
T4	1/352 (0.3)	37/707 (5.2)	NA
Other ^a	24/352 (6.8)	74/707 (10.5)	NA
Tumor size			
Single, ≤1 cm	24/352 (6.8)	86/707 (12.2)	10/44 (22.7)
Single, 1-2 cm	47/352 (13.4)	161/707 (22.8)	9/44 (20.5)
Single, 2-3 cm	27/352 (7.7)	143/707 (20.2)	6/44 (13.6)
Single, >3 cm	58/352 (16.5)	144/707 (20.4)	8/44 (18.2)
Multiple, any size	68/352 (19.3)	139/707 (19.7)	4/44 (9.1)
Unknown	128/352 (36.4)	34/707 (4.8)	7/44 (15.9)

Abbreviations: NA, not available and/or applicable; PUNLMP, papillary urothelial neoplasms of low malignant potential; UC, urothelial carcinoma.
^a Includes patients with UC of unknown pathologic stage and PUNLMP.

at ΔC_t of 10 and 12, respectively, based on the principle of maximizing the Youden index in the area under the receiver operating characteristic curve (eFigure 4 in [Supplement 1](#)).

The assay demonstrated consistently high diagnostic accuracy across both the training and validation cohorts (**Table 2**). Sensitivity exceeded 90% in both cohorts (91.2% [95% CI, 87.6%-93.9%] in cohort 2 and 90.8% [95% CI, 88.4%-92.8%] in cohort 3), while specificity reached 90.0% (95% CI, 85.9%-93.2%) and 93.2% (95% CI, 91.3%-94.7%), respectively. Diagnostic sensitivity was higher than that of either *ALO21918.2* or *VIM* alone (eTable 6 in [Supplement 1](#)). Positive and negative predictive values were estimated across a range of disease prevalences. At an assumed UC prevalence of 15%, the estimated positive predictive value was 70.2% (eTable 7 in [Supplement 1](#)).

Assay sensitivity varied according to tumor stage and grade but was not associated with sex, tumor location, or primary vs recurrent status. In both the training and validation cohorts, specificity for non-UC cancers of the urinary system (primarily kidney and prostate cancers [eTable 8 in

Table 2. Clinical Performance of Study Assay in Training and Validation Cohorts

	Training cohort			Validation cohort		
Characteristic	No. of patients	Performance, % (95% CI)	P value ^a	No. of patients	Performance, % (95% CI)	P value ^a
Sensitivity						
Overall	352	91.2 (87.6-93.9)	NA	707	90.8 (88.4-92.8)	NA
Age, y						
<60	86	82.6 (72.5-89.6)	.001	157	86.0 (79.3-90.8)	.02
≥60	266	94.0 (90.2-96.4)		550	92.2 (89.5-94.2)	
Sex						
Female	81	87.7 (78.0-93.6)	.20	179	91.6 (86.3-95.1)	.77
Male	271	92.3 (88.2-95.0)		528	90.5 (87.6-92.9)	
Location						
Bladder	272	90.1 (85.7-93.2)	.37	486	88.9 (85.7-91.5)	.05
Ureter	27	92.6 (74.3-98.7)		100	92.0 (84.4-96.2)	
Renal pelvis	43	97.7 (86.2-99.9)		94	96.8 (90.3-99.2)	
Urethra	NA			2	100 (19.8-100)	
Concurrent	10	90.0 (54.1-99.5)		25	100 (83.4-100)	
Stage						
Tis	8	100 (59.8-100)	.008	9	88.9 (50.7-99.4)	<.001
Ta	44	79.6 (64.3-89.7)		172	84.3 (77.8-89.2)	
T1	190	95.3 (90.9-97.7)		260	94.6 (90.9-96.9)	
T2-T4	86	96.5 (89.4-99.1)		192	98.4 (95.1-99.6)	
Unknown ^b	12	100 (69.9-100)		55	92.7 (81.6-97.6)	
Primary or not						
Yes	274	91.6 (87.5-94.5)	.61	645	90.9 (88.3-92.9)	>.99
No	78	89.7 (80.3-95.2)		62	90.3 (79.5-96.0)	
Grade						
PUNLMP	12	16.7 (2.9-49.1)	<.001	19	15.8 (4.2-40.5)	<.001
Low grade	88	86.4 (77.0-92.5)		166	82.5 (75.7-87.8)	
High grade	241	96.3 (92.8-98.2)		513	96.1 (93.9-97.5)	
Unknown	11	100 (67.9-100)		9	100 (62.9-100)	
Specificity						
Overall	291	90.0 (85.9-93.2)	NA	913	93.2 (91.3-94.7)	NA
Disease type						
Benign diseases of the urinary tract	186	91.4 (86.2-94.8)	.30	613	92.8 (90.4-94.7)	.006
Non-UC cancer of the urinary system	105	87.6 (79.4-93.0)		122	88.5 (81.2-93.4)	
Nonurinary cancer	NA	NA		178	97.8 (94.0-99.3)	

Abbreviations: NA, not available; PUNLMP, papillary urothelial neoplasms of low malignant potential; UC, urothelial cancer.

^a Calculated using the χ^2 test and Fisher exact test.

^b Refers to patients with unknown pathologic stage, excluding those with PUNLMP.

Supplement 1]) was slightly lower than that for benign diseases of the urinary tract, at 87.6% (95% CI, 79.4%-93.0%) vs 91.4% (95% CI, 86.2%-94.8%) in the training cohort and 88.5% (95% CI, 81.2%-93.4%) vs 92.8% (95% CI, 90.4%-94.7%) in the validation cohort (Table 2). Demographic characteristics of participants and center-specific diagnostic performance are summarized in eTable 9 in Supplement 1.

Detection of Early-Stage and Small Tumors

The methylation assay demonstrated high sensitivity for low-grade and noninvasive UC. The test demonstrated 100% (95% CI, 59.8%-100%) sensitivity for carcinoma in situ in cohort 2 and 88.9% (95% CI, 50.7%-99.4%) sensitivity in cohort 3. The sensitivity for Ta category UC was 79.6% (95% CI, 64.3%-89.7%) in cohort 2 and 84.3% (95% CI, 77.8%-89.2%) in cohort 3, while for low-grade cancer, the sensitivity was 86.4% (95% CI, 77.0%-92.5%) in cohort 2 and 82.5% (95% CI, 75.7%-87.8%) in cohort 3 (Table 2). Among patients with solitary, low-grade, non-muscle-invasive UC lesions of 1 cm or less, the assay detected 32 of 41 tumors (78.0%) (eFigure 5 in Supplement 1). In a patient undergoing transurethral resection for suspected recurrence of bladder tumor due to mucosal congestion identified by white light endoscopy, all 3 noninvasive modalities (magnetic resonance imaging, FISH, and cytology) produced negative results. However, the assay results were positive preoperatively, which was later confirmed by postoperative pathology results (eFigure 5 in Supplement 1).

Comparison of Assay With Cytology and FISH

In the present study, urine cytology was performed on 124 patients with UC. Sample characteristics are provided in eTable 10 in Supplement 1. In head-to-head comparisons, urine cytology showed a specificity of 86.4% (95% CI, 64.0%-96.4%), while the assay showed a specificity of 90.9% (95% CI, 69.4%-98.4%). For UTUC, sensitivity was 44.3% (95% CI, 32.6%-56.6%) for cytology and 97.1% (95% CI, 89.1%-99.5%) for the assay. For bladder cancer, sensitivity was 42.9% (95% CI, 25.0%-62.6%) for cytology and 89.3% (95% CI, 70.6%-97.2%) for the assay (Table 3).

Table 3. Diagnostic Performance of Study Assay vs Cytology or FISH

Characteristic	Cytology vs study assay performance				FISH vs study assay performance			
	No. of patients	Cytology	Assay	P value ^a	No. of patients	FISH	Assay	P value ^a
Sensitivity, % (95% CI)								
Overall UC	102	45.1 (35.3-55.2)	95.1 (88.4-98.2)	<.001	101	61.4 (51.1-70.8)	96.0 (89.6-98.7)	<.001
Location								
Bladder	28	42.9 (25.0-62.6)	89.3 (70.6-97.2)	<.001	39	59.0 (42.2-74.0)	94.9 (81.4-99.1)	<.001
UTUC	70	44.3 (32.6-56.6)	97.1 (89.1-99.5)	<.001	61	62.3 (48.9-74.1)	96.7 (87.6-99.4)	<.001
Concurrent	4	75.0 (21.9-98.7)	100 (39.6-100)	>.99	1	100 (5.5-100)	100 (5.5-100)	>.99
Stage								
Non-muscle-invasive ^b	50	38.0 (25.0-52.8)	94.0 (82.5-98.4)	<.001	56	58.9 (45.0-71.6)	94.6 (84.2-98.6)	<.001
Muscle invasive ^c	44	54.5 (39.0-69.3)	97.7 (86.5-99.9)	<.001	36	72.2 (54.6-85.2)	97.2 (83.8-99.9)	.003
Unknown	8	37.5 (10.2-74.1)	87.5 (46.7-99.3)	.12	9	33.3 (9.0-69.1)	100 (62.9-100)	.009
Grade								
Low	16	18.8 (5.0-46.3)	81.3 (53.7-95.0)	.001	13	38.5 (15.1-67.7)	84.6 (53.7-97.3)	.04
High	80	52.5 (41.1-63.7)	98.8 (92.3-99.9)	<.001	77	70.1 (58.5-79.8)	97.4 (90.1-99.6)	<.001
Unknown	6	16.7 (0.9-63.5)	83.3 (36.5-99.1)	.008	11	27.3 (7.3-60.7)	100 (67.9-100)	.001
Specificity, % (95% CI)								
Overall interfering disease	22	86.4 (64.0-96.4)	90.9 (69.4-98.4)	>.99	17	76.5 (49.8-92.2)	94.1 (69.2-99.7)	.34

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

^b Includes Tis, Ta, and T1 categories.

^c Includes T2, T3, and T4 categories.

^a Calculated using the χ^2 test and Fisher exact test.

Among 118 patients who underwent both FISH and assay testing, FISH showed a specificity of 76.5% (95% CI, 49.8%-92.2%), whereas the assay showed a specificity of 94.1% (95% CI, 69.2%-99.7%). For UTUC, sensitivity was 62.3% (95% CI, 48.9%-74.1%) for FISH and 96.7% (95% CI, 87.6%-99.4%) for the assay. For bladder cancer, the sensitivity was 59.0% (95% CI, 42.2%-74.0%) for FISH and 94.9% (95% CI, 81.4%-99.1%) for the assay (Table 3). Compared with urine cytology and FISH, the assay demonstrated higher sensitivity for UC while maintaining comparable or higher specificity.

Assay Performance in UC Recurrence Surveillance

Among 33 paired preoperative and postoperative samples in cohort 3, methylation clearance was observed in 30 of 32 patients (93.8%) with positive preoperative assay results after surgery (eFigure 6 in Supplement 1). In cohort 4, 9 patients experienced clinical recurrence during surveillance, and the assay detected all recurrence events (median follow-up time, 6 [range, 2-11] months) (Figure 2). The median lead time of assay positivity before clinical confirmation of recurrence was 4.5 (IQR, 1.5-5.5) months (eFigure 7 in Supplement 1).

Among 35 patients without clinical recurrence, assay results remained negative in 29 (82.9%) (Figure 2). Eight patients exhibited positive assay results within 1 month after surgery (eFigure 7 in Supplement 1). Comparison of ΔC_t values between preoperative and postoperative samples showed decreased methylation levels, particularly for *VIM*. Additionally, 2 patients (P008 and P020) had at least 2 positive assay results during follow-up. However, no clinical recurrence was observed in these patients during the available follow-up period.

Discussion

In this large prospective diagnostic study, we found that a urinary methylation assay demonstrated high sensitivity and specificity for UC detection. Moreover, the assay showed higher sensitivity than urine cytology and FISH while maintaining high specificity. Notably, the assay enables simultaneous detection of both bladder cancer and UTUC. UTUC is frequently diagnosed at an advanced stage, partly because localized disease is often clinically silent.¹⁸ Moreover, the anatomical inaccessibility of the upper urinary tract makes endoscopic evaluation challenging.¹⁹ These limitations highlight the need for accurate, noninvasive biomarkers that can facilitate earlier detection of UTUC.

Urinary DNA methylation assays have emerged as promising tools for UC diagnosis and surveillance. Previous urinary assays, including EpiCheck,¹⁴ Bladder CARE,²⁰ utMeMA,²¹ OncoUrine,²² and P3 panel,²³ have shown variable diagnostic performance (eTable 11 in Supplement 1). Compared with previous methylation studies, the present study included one of the largest prospective cohorts to date and additionally evaluated postoperative surveillance, extending the potential clinical application beyond initial diagnosis. Furthermore, many comparable assays require substantial urine volumes and specialized centrifugation, which may limit their routine clinical implementation.²⁴ Conversely, the present assay requires only 1 mL of whole urine without centrifugation, providing a simplified workflow that may facilitate future clinical application.

While most UC cases present with hematuria, most cases of hematuria are caused by benign urologic conditions or other urologic cancers.²⁵ Therefore, high specificity is an important consideration when evaluating urinary biomarkers in this clinical setting. This assay demonstrated 92.8% specificity for benign urologic diseases and modestly lower specificity in patients with other genitourinary malignant neoplasms; test results must be interpreted in the context of each patient's full clinical profile. Although a modest decrease in specificity was observed in non-UC cancers such as kidney or prostate cancer, these entities are generally distinguishable from UC based on imaging and anatomical localization, minimizing the clinical impact of potential false-positive findings.

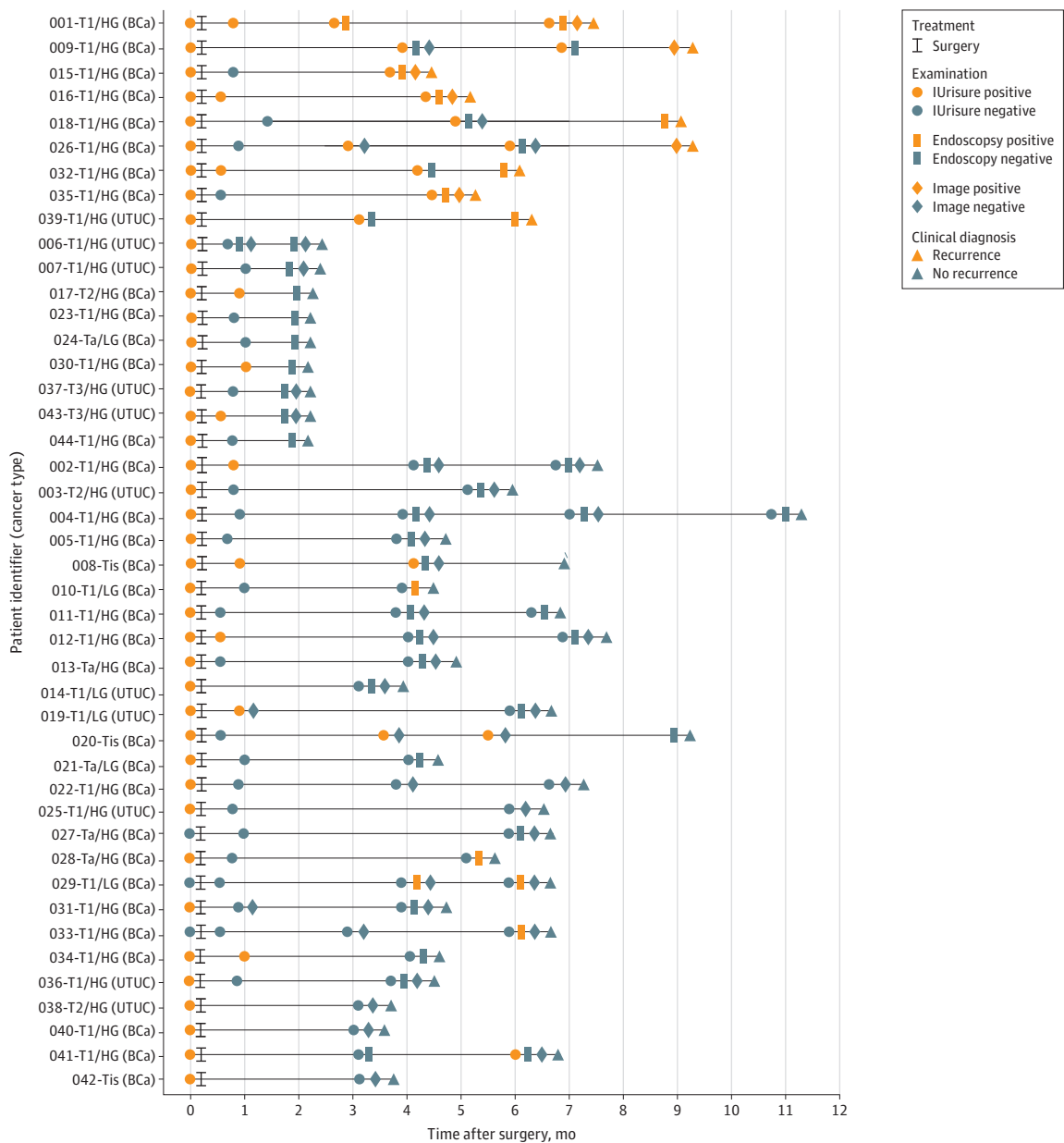
During follow-up, negative assay results were observed in most patients with UC postoperatively. However, a positive assay result after surgery may indicate the presence of minimal residual disease and an elevated risk of UC recurrence. Notably, the assay demonstrated a median

lead time of 4.5 months in detecting recurrence before clinical manifestation. This finding suggests that a positive assay result, even in the absence of cystoscopic or imaging evidence, should prompt intensified surveillance with shortened follow-up intervals to enable earlier detection and timely intervention for recurrent disease. Given the high recurrence rates of UC, earlier identification of recurrence may support more effective surveillance strategies.

Limitations

This study has some limitations. Although urinary molecular markers have not yet been fully adopted into clinical guidelines as stand-alone tools for the routine diagnosis or follow-up of UC, major guidelines acknowledge their potential value as complementary methods.^{26,27} In this context,

Figure 2. Dot Plot Showing Longitudinal Representation of the Study Assay, Endoscopy, and Image Results for Each Patient



Patient identifiers consist of a sequential study enrollment number assigned by time of study entry, followed by tumor stage and grade. BCa indicates bladder cancer; HG, high grade; LG, low grade; and UTUC, upper tract urothelial carcinoma.

urinary methylation markers may help to rule in patients for cystoscopy during initial detection or rule out recurrence during surveillance. High-quality randomized clinical trials comparing assay-assisted with standard cystoscopic surveillance are necessary to validate the assay's clinical utility. Moreover, the surveillance cohort was relatively small. The relatively short follow-up duration may lead to underestimation of specificity, as some current false-positive findings might represent early molecular change prior to clinical recurrence. Extending the follow-up period is therefore expected to further improve the accuracy of the present assay in recurrence monitoring. Further large-scale, dedicated longitudinal surveillance studies are needed to validate the accuracy of the assay for monitoring UC recurrence.

Conclusions

In this multicenter diagnostic study, the present assay demonstrated high diagnostic accuracy, with a sensitivity of 90.8% and specificity of 93.2%, and showed higher sensitivity than urine cytology and FISH in comparative analyses. Furthermore, the assay detected recurrence-associated methylation changes with a median lead time of 4.5 months before clinical confirmation. These findings support the use of this assay as a complementary, noninvasive tool for UC diagnosis and postoperative surveillance.

ARTICLE INFORMATION

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SUPPLEMENT 1.

eMethods. Materials and Participants

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eFigure 3. The Methylation Levels Analysis in Cohorts 1 and 2

eFigure 4. Cutoff Determination of *ALO21918.2* and *VIM* Methylation Markers

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eFigure 7. Application of Assay to Monitor Recurrence in UC During Follow-Up

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eTable 11. Comparison of Study Assay With Other Noninvasive Methylation Tests for the Diagnosis of UC in Published Studies

SUPPLEMENT 2.

Data Sharing Statement