Comparison of 2 New Real-Time Polymerase Chain Reaction–Based Urinary Markers in the Follow-Up of Patients With Non–Muscle-Invasive Bladder Cancer

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BACKGROUND: The objective of the current study was to compare the diagnostic accuracy of 2 new real-time polymerase chain reaction–based urinary markers with each other and with urinary cytology, cystoscopy, and/or histology in patients being followed for non–muscle-invasive bladder cancer. **METHODS:** A total of 487 patients were enrolled in the study. Patients were evaluated using voided urine cytology, the Xpert Bladder Cancer Monitor, the Bladder EpiCheck test, and white light cystoscopy. **RESULTS:** The overall sensitivity was 27.17% for cytology, 64.13% for the Bladder EpiCheck test, and 66.3% for the Xpert Bladder Cancer Monitor. The overall specificity was 98.82% for cytology, 82.06% for the Bladder EpiCheck test, and 76.47% for the Xpert Bladder Cancer Monitor. The negative predictive value was very similar for the 3 tests at 83.56% for cytology, 89.42% for the Bladder EpiCheck test, and 89.35% for the Xpert Bladder Cancer Monitor. When combined, the Bladder EpiCheck test and Xpert Bladder Cancer Monitor detected overall 79.35% of the tumors: 70.37% in low-grade and 92.11% in high-grade tumors. **CONCLUSIONS:** The Xpert Bladder Cancer Monitor and Bladder EpiCheck test were found to perform very well in terms of sensitivity. Together, the 2 tests detected approximately 92.11% of high-grade tumors. Their specificity was high but could not reach the excellent value of cytology. The negative predictive value was the same for both tests and was higher than that for cytology, especially when the tests were used together (92.24%). These 2 new tests hold promise as urinary biomarkers. They may be used in combination to maximize sensitivity in a less invasive way, thereby reducing invasiveness in the follow-up of patients with non–muscle-invasive bladder cancer and decreasing discomfort for the patients as well as complications and costs. ***Cancer Cytopathol* 2020;0:1-7**. © *2020 American Cancer Society*.

KEY WORDS: bladder cancer; cytology; follow-up; non–muscle-invasive bladder cancer; urinary marker.

INTRODUCTION

Non–muscle-invasive bladder cancer (NMIBC) represents approximately 70% to 80% of newly diagnosed urothelial bladder cancer (BC) cases in the industrialized world.[1] Of these patients, approximately 50% develop a recurrence within 5 years, 25% experience disease progression, and 10% to 15% of patients will die of the disease.[2]

The surveillance of patients with a history of NMIBC is performed using cystoscopy and urinary cytology. Cystoscopy is an invasive procedure and easy to perform, but can cause complications, anxiety, and significant discomfort to the patient; its sensitivity is high for papillary lesions but not for flat ones

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and it requires experience. Urinary cytology commonly is used in addition to cystoscopy, especially to discover flat lesions, which cannot be detected by cystoscopy. Although it has a high sensitivity for high-grade (HG) tumors, its sensitivity is low for low-grade (LG) tumors.[3,4] Furthermore, chronic urinary tract infections, stone disease, or benign prostatic hyperplasia can lead to degenerative cellular changes or atypia, which can reduce the sensitivity of urinary cytology. The same is true for intravesical therapy in patients with HG tumors.[5]

The side effects and costs associated with the limitations of cytology and cystoscopy in the surveillance of patients after NMIBC have led to decades of research regarding urinary markers for the early detection of BC. The majority of these urinary markers are more sensitive than cytology in LG and HG tumors but their specificity remains significantly lower. To our knowledge, to date none are able to replace cytology and cystoscopy and have not been introduced into clinical practice.[6,7]

Recently, 2 new real-time polymerase chain reaction (PCR)–based urinary markers have been tested. The Bladder EpiCheck test is based on DNA methylation changes associated with BC, which appears to be related to progression of NMIBC to muscle-invasive BC,[8] and analyzes a panel of 15 methylation biomarkers.[9]

The Xpert Bladder Cancer Monitor measures the level of 5 target messenger RNAs (mRNAs), which appear to be upregulated in urine specimens from subjects with BC.[10] In 2 previous independent studies, we evaluated the performance of each single test on different patient cohorts and compared them with cytology, cystoscopy, and/or histology.[11,12]

The objective of the current study was to compare the diagnostic accuracy of these 2 new urinary markers with each other and with urinary cytology, cystoscopy, and/or histology in the same cohort of patients being followed for NMIBC.

MATERIALS AND METHODS

After approval of the local institutional ethics committee (47-2017 and 37-2018) and obtaining informed consent, a total of 487 patients who were being followed for NMIBC were enrolled consecutively in the current prospective study. The median age of the patients (379 of whom were male and 108 of whom were female) was 74 years (SD, 9.51 years). Of the 487 patients enrolled in the current study, at the time of first diagnosis, 215 patients (44.15%) were classified as having pTa grade 1 disease, 102 (20.95%) were classified as having pTa grade2 disease, 19 (3.90%) were classified as having pT1 grade 2 disease, 24 (4.93%) were classified as having pTa grade 3 disease, 68 (13.96%) were classified as having pT1 grade 3 disease, and 59 (12.11%) patients were classified as having carcinoma in situ.

A total of 122 patients (25.05%) were treated with intravesical therapy with bacille Calmette-Guérin and 37 (7.6%) with mitomycin.

Patients were followed using voided urine cytology and with white light cystoscopy, according to the current European Association of Urology guidelines,[13] and by the mRNA-based Xpert Bladder Cancer Monitor and the methylation-based Bladder EpiCheck test. We reserved photodynamic cystoscopy for those patients with positive cytology and no visible bladder tumor. In the current study, none of the patients underwent photodynamic cystoscopy in an outpatient setting.

Any lesion consider to be suspicious on cystoscopy was biopsied or removed transurethrally and specimens were evaluated according to the 2017 TNM classification of urinary bladder cancer and graded according to both the 1973 and the 2004 World Health Organization grade classifications.[14,15] Patients were classified as negative when white light cystoscopy, cytology, and histology were negative.

From the voided urine of each patient, a total of 20 to 30 mL were added to 15 mL of CytoLyt fixation liquid (Hologic Inc, Marlborough, Massachusetts) in a Falcon tube for urinary cytology and sent to the laboratory along with a minimum of 12 mL of fresh voided urine for the Bladder EpiCheck test and 4.5 mL of urine, added to the Xpert Transport kit, containing an RNA stabilizing solution.

Cytology

The Falcon tubes were centrifuged for 10 minutes at 600 × g. The resulting cell pellets were resuspended in ThinPrep vials containing methanol-based PreservCyt solution and processed using the ThinPrep 5000 System (Hologic Inc). Cytological specimens were stained according to the Papanicolaou staining procedure.[11,12] Cytological diagnosis was performed according to The Paris System for Reporting Urinary Cytology,[16] classifying the cytological specimens accordingly into negative

for HG urothelial carcinoma, atypical urothelial cells, suspicious for HG urothelial carcinoma, HG urothelial carcinoma, LG intraepithelial neoplasia, or not diagnostic.

For the statistical analysis, negative for HG urothelial carcinoma and atypical urothelial cells were grouped as negative, and suspicious for HG urothelial carcinoma, HG urothelial carcinoma, and LG intraepithelial neoplasia were grouped as positive.

Xpert Bladder Cancer Monitor Test

The Xpert Bladder Cancer Monitor test (Cepheid Srl, Milan, Italy) measures the level of 5 target mRNAs (ABL1, CRH, IGF2, UPK1B, and ANXA10) in 4.5 mL of voided, stabilized urine using real-time, reverse transcriptase–PCR in a prefilled cartridge. The results are interpreted using the GeneXpert Instrument System from measured fluorescent signals and embedded calculation algorithms. The test result, laboratory developed assay (LDA) totals, and analyte results are shown in the test report. A cutoff value was set at an LDA of >0.5.[12]

Bladder EpiCheck Test

As previously reported by Trenti et al,[11] when using the Bladder EpiCheck test (Nucleix Ltd, Rehovot, Israel), the urine sample was centrifuged twice at 1000 × g for 10 minutes at room temperature. DNA was extracted from the cell pellet using the Bladder EpiCheck DNA extraction kit. The extracted DNA was digested using a methylation-sensitive restriction enzyme that cleaves DNA at its recognition sequence if it is unmethylated. The quantitative PCR amplification was performed on the digested DNA using the Rotor-Gene Q instrument (Qiagen, Venlo, the Netherlands). The samples were prepared for the PCR assay using the Bladder EpiCheck test kit and the amplification data were analyzed using the Bladder EpiCheck software. For the samples that passed the internal control validation, the software calculated an EpiScore (a number between 0 and 100) representing the overall methylation level of the sample at the panel biomarkers. An EpiScore of ≥60 indicates a positive result, whereas a score <60 indicates a negative result.

Statistical Analysis

The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of cytology, the Bladder EpiCheck test, and the Xpert Bladder Cancer Monitor versus histology and/or cystoscopy were calculated. Statistical analysis was performed and the area under the receiver operating characteristic curve (AUC) was calculated as well as ancillary metrics. The DeLong test was performed to assess statistical differences between the 2 receiver operating characteristic curves.[17] A regularized logistic regression[18] was used to create a score that optimally combines the Bladder EpiCheck test, the Xpert Bladder Cancer Monitor, and the Youden approach[19] to obtain the optimal cutoff value. The R Statistical environment was used for the statistical analysis and the significance threshold was set as an α of .05.

TABLE 1. Demographic and Clinical Characteristics of 487 Patients

Characteristic	Value	
Median age, y	74 ± 9.51	
Sex, no. (%)		
Male	379	77.8
Female	108	22.2
First diagnosis, no. (%)		
LG	336	69
HG	151	31
Previous BCG	122	25.1
Previous MMC	37	7.6
Excluded due to error sign in Bladder EpiCheck test	41	8.4
Excluded due to invalid/error Xpert Bladder Cancer Monitor	14	2.9

Abbreviations: BCG, bacille Calmette-Guérin; HG, high grade; LG, low grade; MMC, mitomycin.

RESULTS

Of the 487 patients, 55 (11.3%) had to be excluded because of an invalid test: 14 from the Xpert Bladder Cancer Monitor test (2.9%) and 41 (8.4%) from the Bladder EpiCheck test. Of the 432 remaining patients, 92 (21.3%) demonstrated NMIBC recurrence, including 54 patients with LG NMIBC (58.7%) and 38 with HG NMIBC (41.3%). Demographic and clinical characteristics of the patients are summarized in Table 1.

The overall sensitivity was 27.17% for cytology, 64.13% for the Bladder EpiCheck test, and 66.3% for the Xpert Bladder Cancer Monitor test. The sensitivity of cytology increased from 12.96% in LG tumors to 47.37% in HG tumors, whereas for the Bladder EpiCheck test, the sensitivity was 53.7% in LG tumors and 78.95% in HG tumors and was 57.41% for LG tumors and 78.95% for HG tumors when using the Xpert Bladder Cancer Monitor.

The overall specificity was 98.82% for cytology, 82.06% for the Bladder EpiCheck test, and 76.47% for the

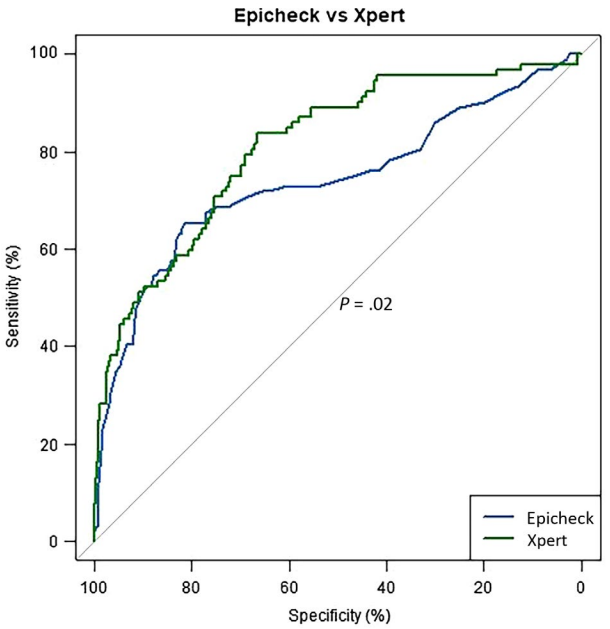

Figure 1. Area under the curve for all patients irrespective of the tumor grade.

Xpert Bladder Cancer Monitor. The PPV for cytology was 86.21%, that for the Bladder EpiCheck test was 49.17%, and the PPV for the Xpert Bladder Cancer Monitor was 43.26%. The NPV was very similar for the 3 tests: 83.56% for cytology, 89.42% for the Bladder EpiCheck test, and 89.35% for the Xpert Bladder Cancer Monitor.

Both tests demonstrated a high diagnostic efficacy, with the Bladder EpiCheck test found to have an AUC of 73.8% (95% CI, 67.2%-80.5%) and the Xpert Bladder Cancer Monitor found to have an AUC of 81% (95% CI, 75.9%-86.2%). A significant difference between the accuracy of both tests was observed in the final statistical analysis (DeLong method $P = .002$) (Fig. 1).[17]

Evaluating both tests and considering the result as positive if 1 of the 2 tests was positive, the Bladder EpiCheck test and Xpert Bladder Cancer Monitor overall were found to detect approximately 79.35% of the tumors (73 of 92 tumors). When calculated according to grade, the combined sensitivity was 70.37% in LG and 92.11% in HG tumors. The specificity of both tests together was 66.47%, the PPV was 39.04%, and the NPV was 92.24%. Detailed data are shown in Tables 2 and 3.

Subsequently, the 2 tests were combined using a regularized logistic regression with the objective of achieving a synthetic score and returned an AUC of 83% (Figs. 2 and 3). A sensitivity of 70% and a specificity of 86% were obtained using a cutoff value of 0.27 of the synthetic score.

DISCUSSION

BC is the 7th most commonly diagnosed cancer worldwide in the male population (11th if both sexes are considered). Approximately 75% of patients are diagnosed with early-stage NMIBC.[13] The high rate of recurrence and the risk of disease progression in approximately 25% of the cases suggest the need for life-long monitoring with frequent cystoscopies, which are invasive, cause discomfort to the patient, and reduce patient compliance with follow-up regimens.[20]

Conventional white light cystoscopy is not able to detect 10% to 20% of tumors, the majority of which are flat lesions. Moreover, in approximately 10% of patients, a urinary tract infection can occur as a complication.[21] Urine cytology commonly is used as an adjunct to cystoscopy. Although it is very useful in patients with HG tumors, its sensitivity in LG tumors is very low. Furthermore, an experienced cytopathologist is needed to avoid misinterpretation.[22] Therefore, several potential urine-based markers have been developed over the last decades because of the necessity to improve the low sensitivity of cytology, but to the best of our knowledge none of these unfortunately was suitable for application in clinical practice.

Recently, 2 new promising markers were introduced to the diagnostics of BC: the Xpert Bladder Cancer Monitor and the Bladder EpiCheck test.

The accuracy of the Xpert Bladder Cancer Monitor first was evaluated by Pichler et al in a series of 140 patients, with an overall sensitivity of 84% reported for the Xpert Bladder Cancer Monitor and 33% for bladder washing cytology[10] and by Van Valenberg et al in a multicenter study with 255 patients, which reported a lower sensitivity, with 75% noted for the Xpert Bladder Cancer Monitor and 29.5% for washing cytology.[23] In our previous study, we analyzed a total of 230 patients, and reported an overall sensitivity of 46.2% for the Xpert Bladder Cancer Monitor and 11.5% for voided urinary cytology.[12]

The Bladder EpiCheck test was validated by Wasserstrom et al in a series of 222 patients with NMIBC who were under surveillance, and demonstrated a sensitivity of 90% and a specificity of 83%, with an NPV of 97%.[9] In 2 other recent studies by Witjes et al[24] and D'Andrea et al,[25] both of which

TABLE 2. Overall Sensitivity, Specificity, PPV, and NPV of Cytology, Bladder EpiCheck Test, Xpert Bladder Cancer Monitor, and the Combination of the 2 Tests in 432 Evaluable Patients

	Cytology (95% CI), %	Bladder EpiCheck (95% CI), %	Xpert Bladder Cancer Monitor (95% CI), %	Bladder EpiCheck and Xpert Bladder Cancer Monitor (95% CI), %
Sensitivity	27.17 (18.42-37.45)	64.13 (53.46-73.87)	66.30 (55.70-75.83)	79.35 (69.64-87.08)
Specificity	98.82 (97.02-99.68)	82.06 (77.56-85.99)	76.47 (71.59-80.88)	66.74 (61.18-71.47)
PPV	86.21 (69.05-94.60)	49.17 (42.38-55.99)	43.26 (37.48-49.24)	39.04 (34.79-43.45)
NPV	83.56 (79.73-86.94)	89.42 (86.49-91.78)	89.35 (86.22-91.83)	92.24 (88.78-94.70)

Abbreviations: NPV, negative predictive value; PPV, positive predictive value.

TABLE 3. Sensitivity of the Tests According to Grade in 92 Tumors

	Cytology (95% CI), %	Bladder EpiCheck (95% CI), %	Xpert Bladder Cancer Monitor (95% CI), %	Bladder EpiCheck and Xpert Bladder Cancer Monitor (95% CI), %
LG (54)	12.96 (5.37-24.9)	53.70 (39.61-67.38)	57.41 (43.21-70.77)	70.37 (56.39-82.02)
HG (38)	47.37 (30.98-64.18)	78.95 (62.68-90.45)	78.95 (62.68-90.45)	92.11 (78.62-98.34)

Abbreviations: HG, high grade; LG, low grade.

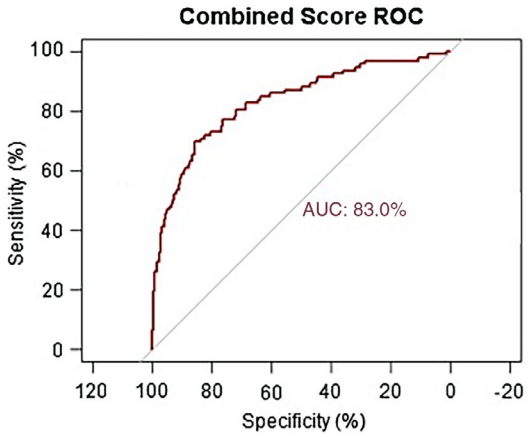

Figure 2. Area under the curve (AUC) of both tests combined. ROC indicates receiver operating characteristic curve.

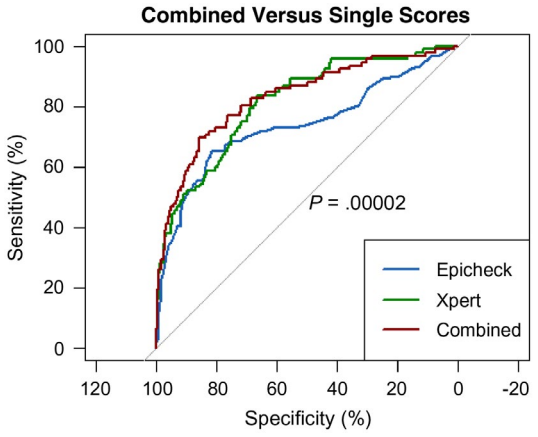

Figure 3. Area under the curve of the Bladder EpiCheck test, Xpert Bladder Cancer Monitor, and the combination of both tests.

included 440 patients, similar overall sensitivities of 68.2% and 67.3%, respectively, were demonstrated and a specificity of 88.0% with a dropout rate of 8.4%.[24] In our previous study, we analyzed a preliminary series of 243 patients who were being followed for NMIBC and reported an overall sensitivity of 62.3% for the Bladder EpiCheck test and 33.3% for voided urinary cytology, with a dropout rate of 11.5%.[11]

In the current study, we evaluated the diagnostic accuracy of both tests prospectively in the same patient cohort under surveillance for NMIBC, and compared the results with those of cytology.

The dropout rate in the current study decreased to 8.4% for the Bladder EpiCheck test and was 2.9% for Xpert Bladder Cancer Monitor. Although the Bladder EpiCheck test requires experienced technicians and a molecular pathology laboratory and has a long learning curve, the Xpert Bladder Cancer Monitor is easy to perform and does not need a professional technician.

The 2 tests performed very well in terms of overall sensitivity, with a sensitivity of 66.3% reported for the Xpert Bladder Cancer Monitor and 64.13% for the Bladder EpiCheck test. In contrast, the overall sensitivity of cytology was low at 27.17% ($P < .0001$). The sensitivity of the 2 tests in the current study cohort was higher than in our previous cohorts,[11,12] but was lower than in the previous studies published by the other study groups.[10,23-25] The sensitivity increased to 78.95% for both tests when calculated only for HG tumors whereas the sensitivity of cytology rose to only 47.37% in HG tumors in the current study cohort. However, when combining the 2 markers, the overall sensitivity increased to 79.35% and to 92.11% when calculated

for HG tumors only. Comparing the significantly higher sensitivity of the Xpert Bladder Cancer Monitor and the Bladder EpiCheck test and the 2 tests combined with cytology, the tests appeared to be a valid tool for improving the early diagnosis of BC recurrence. When combined with cytology, one can take advantage of its high specificity, which could not be reached using the 2 markers. In fact, the overall specificity of the 2 tests was good, at 76.47% for the Xpert Bladder Cancer Monitor and 82.06% for the Bladder EpiCheck test, but was markedly lower than for cytology (98.82%). Compared with the previous studies, specificity was the same for the Xpert Bladder Cancer Monitor (76.47% vs 77%)[12] and was considerably lower for the Bladder EpiCheck test (76.47% vs 86.3%).[11] Compared with the data published by the other groups,[10,23-25] the specificity in the current study was lower for the 2 tests. The lower specificity could be the result of false-positive results in patients undergoing instillation therapy and in the case of anticipatory positive results, as already has been discussed previously.[12] Patients who undergo instillation therapy still could have a genetic instability at a time when voided urinary cytology does not yet demonstrate any atypia and other patients may demonstrate genetic alterations due to tumor recurrence, which cannot yet be observed by cytology.[12]

Consequently, the PPV in the current study was 2-fold higher for cytology (86.21%) compared with the Xpert Bladder Cancer Monitor (43.26%) and the Bladder EpiCheck test (49.17%). The results were the same when combining both tests. However, the NPV was 82.56% for cytology and was higher and very similar for the Xpert Bladder Cancer Monitor and Bladder EpiCheck test (89.35% vs 89.42%), reaching 92.24% when these tests were combined. However, the high NPV reported in the previous studies[9,10,23-25] could not be achieved.

In terms of diagnostic accuracy, we found a significant difference by comparing the 2 tests, with an AUC of 81% for the Xpert Bladder Cancer Monitor and 73.8% for the Bladder EpiCheck test. Both tests performed very well with a high diagnostic efficacy, reaching a sensitivity of 92.11% for HG tumors and an NPV of 92.24% if used together.

An important point to be remembered is the cost. The ideal urinary marker is highly sensitive and specific, easy to perform, and inexpensive. These 2 new urinary markers are not inexpensive. In the study institution, both tests together cost €250, whereas voided urine thin-layer cytology costs €25 euro, which is 10 times less. In contrast, 1 cystoscopy costs €400. Considering the high sensitivity of the 2 tests combined and the high specificity of cytology, as well as the invasiveness and low sensitivity of cystoscopy in patients with flat lesions, one could consider performing both tests along with cytology and therefore reduce the number of cystoscopies provided that both tests and the cytology result are negative, not only in low-risk patients but also in the follow-up of patients with HG tumors given the high NPV achieved by combining 2 tests.

Conclusions

The Xpert Bladder Cancer Monitor and Bladder EpiCheck test performed very well in terms of sensitivity, with results that were significantly higher than that for cytology in both LG and HG tumors, particularly when used together. Together, the 2 tests detected approximately 92.11% of HG tumors. Their specificity was high but could not reach the excellent value of cytology. The NPV was the same for both tests and was higher than that for cytology, especially when the tests were used together (92.24%).

These 2 new tests are promising as urinary biomarkers. They may be used in combination to maximize sensitivity in a less invasive way by reducing invasiveness in the follow-up of patients with NMIBC and decreasing discomfort for the patients as well as complications and costs.

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CONFLICT OF INTEREST DISCLOSURES

The authors made no disclosures.

AUTHOR CONTRIBUTIONS

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