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Bladder Cancer



A Novel Urine-Based Assay for Bladder Cancer Diagnosis: Multi-Institutional Validation Study

Noa Davis^{a,*}, Alexander Shtabsky^b, Sylvia Lew^c, Ronny Rona^d, Ilan Leibovitch^e, Ofer Nativ^f, Michael Cohen^g, Yoram Mor^h, Uri Lindnerⁱ, Yael Glickman^a, Haim Matzkin^j, Alexander Tsivian^k, Ofer Gofrit¹, Ofer Yossepovitch^m

^a Micromedic Technologies Ltd., Tel Aviv, Israel; ^b Institute of Pathology, Tel Aviv Sourasky Medical Center, Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel; ^c Patho-Lab Diagnostics Ltd., Ness Ziona, Israel; ^d Kfar Saba, Israel; ^e Department of Urology, Meir Medical Center, Kfar Saba, Israel; ^f Department of Urology, Bnai Zion Medical Center, Haifa, Israel; ^g Department of Urology, HaEmek Medical Center, Afula, Israel; ^h Department of Urology, Sheba Medical Center, Tel Aviv, Israel; ⁱ Department of Urology, Kaplan Medical Center, Rehovot, Israel; ^j Department of Urology, Sourasky Medical Center, Tel Aviv, Israel; ^k Department of Urology, Wolfson Medical Center, Holon, Israel; ¹ Department of Urology, Hadassah Medical Center, Jerusalem, Israel; ^m Department of Urology, Rabin Medical Center, Petach Tikva, Israel

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Abstract

Background: CellDetect is a unique histochemical stain enabling color and morphological discrimination between malignant and benign cells based on differences in metabolic signature. **Objective:** The objective of the present study was to validate the performance of this assay in a controlled, blinded, multicenter study.

Design, setting, and participants: The study, conducted in nine hospitals, included patients with documented history of bladder cancer, monitored for urothelial carcinoma (UCC) or scheduled for bladder cancer surgery.

Outcome measurements and statistical analysis: Cystoscopy and/or biopsy were used as a reference standard to determine sensitivity and specificity. Smears were stained by CellDetect and interpreted by two cytologists blinded to the patient's final diagnosis. The findings were compared with those of standard urine cytology and BTA stat.

Results and limitations: Two hundred and seventeen voided urine specimens were included. Ninety-six (44%) were positive by histology and 121 (56%) were negative by either cystoscopy or histology. The overall sensitivity of CellDetect was 84%. Notably, the sensitivity for detecting low-grade nonmuscle-invasive bladder cancer tumors was greater than this of BTA stat (78% vs 54%) and more than two-fold higher compared with standard cytology (33%, $p \le 0.05$). The specificity was 84% in patients undergoing routine surveillance by cystoscopy. At a median follow-up of 9 mo, 21% of the patients with positive CellDetect and negative reference standard developed UCC, which was significantly higher compared with the 5% of the true negative cases. Limitations include the lack of instrumental urine samples and the lack of patients with nongenitourinary cancers in the study population.

Conclusions: This study validates the performance of CellDetect as a urine-based assay to identify UCC in patients with history of bladder cancer. The high sensitivity was maintained across all cancer grades and stages without compromising the assay specificity. Further studies are required to test whether this novel stain can be incorporated in routine bladder cancer surveillance as a noninvasive alternative to cystoscopy.

Patient summary: Surveillance of bladder cancer requires frequent invasive procedures. In the present study, we validate the ability of a novel biomarker to accurately identify early-stage tumors in urine specimens for the noninvasive monitoring of patients with history of bladder cancer.

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* Corresponding author. Micromedic Technologies Ltd., Kiryat Atidim, Tel Aviv 6158101, Israel. Tel. +972-732753427; Fax: +972-73-2753451. E-mail address: noa@m-medic.com (N. Davis).

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1. Introduction

Urothelial cell carcinoma (UCC) is the most common malignancy in the urinary system [1], with a worldwide prevalence of 2.7 million patients [2] and an annual incidence of 429 800 new cases [3]. With up to an 80% recurrence rate, UCC often requires a lifelong routine surveillance depending on disease severity [4], and is thus considered one of the most costly cancers in terms of lifetime expenditure per patient [5,6]. Cystoscopy remains the reference standard for UCC diagnosis and management, notwithstanding its invasive nature and relatively high cost [7]. The need for noninvasive, accurate, cost-effective markers for UCC surveillance is evident.

Urine cytology is the most frequently used, noninvasive assay for UCC detection [8]. However, its low sensitivity, particularly in low-grade tumors, remains a major hurdle [9]. Over the past 2 decades, additional urinary markers have been developed [10-12]; however, none of them have been proven sufficiently accurate and cost-effective to be integrated in routine patient management [13].

CellDetect is a novel histochemical staining platform allowing color and morphological discrimination between normal and neoplastic cells [14,15]. The discriminative capacity of the stain is assumingly related to the increased metabolic activity inherent in cancer cells. This so called Warburg Effect, characterized by the aerobic glycolysis of malignant tumors, is commonly used in cancer diagnostics such as tumor imaging using labeled glucose analogues (ie, positron emission tomography-computed tomography).

CellDetect is composed of a unique plant extract (*Ficus elastica*) and generic dyes. The active component of the plant extract was found to be a member of the proantocyanidin family, a class of polyphenols present in a variety of plants. While the molecular mechanism is not yet fully elucidated, a series of experiments performed on multiple cell lines showed that the purified active component interacts specifically with proteins found in malignant cells, thus leading to their specific labeling with the red dye. Benign epithelial cells are counter-stained in green (Fig. 1). In addition, functional testing showed that the isolated molecule successfully replaces the crude extract (unpublished data).

CellDetect has been validated in multiple types of cancers in both histological and cytological preparations [16–18]. Recently, the technology has been implemented in a preliminary study of UCC, demonstrating high sensitivity in detecting both low- and high-grade tumors [19]. The present multicenter trial was designed to validate these findings and further explore its utility in patients undergoing routine bladder cancer monitoring.

2. Patients and methods

2.1. Study design

This was a controlled, blinded, multi-center, longitudinal trial involving nine medical centers in Israel, conducted according to good clinical practice, the Declaration of Helsinki, and the requirements and regulations of the Israeli Ministry of Health. Adult patients monitored for UCC were consecutively enrolled if they had a documented history of UCC, if at least 4 wk had passed since any treatment or procedure for UCC was performed, and if they were able to provide a spontaneous urine sample. Patients with catheters, neobladder, ileal conduit, or kidney stones, patients under any other cancer treatment, or patients with suspicious/positive cystoscopy without a subsequent biopsy were excluded. No further selection was performed in these patients.

Voided urine samples were collected from a first cohort of patients undergoing routine cystoscopic surveillance. To enrich the study with positive cases, a second cohort of patients, scheduled for transurethral resection (TURBT) or radical cystectomy, was enrolled as well. The patients from both cohorts had a documented history of bladder cancer.

A minimum of 50-ml voided urine was collected from each participant. For urinalysis, Multistix 10SG (Siemens Healthcare GmbH, Erlangen, Germany) was used. Fresh specimens were tested with the predicate Food and Drug Administration-approved BTA stat (Polymedco Inc., Cortlandt Manor, NY, USA) according to manufacturer's instructions and an aliquot was separated subsequently for standard urine cytology. The remaining urine volume was treated with a designated fixative and preserved at 4 °C. Samples were then processed to cytocentrifuge smears in a central lab and fixed in 96% ethanol.

2.2. Staining

Smears were stained by CellDetect according to manufacturer's instructions (Zetiq Technologies Ltd., Tel Aviv, Israel). Briefly, the CellDetect kit contains a proprietary plant extract and two histological dyes. The staining procedure includes fixation with 10% trichloroacetic acid, nuclear staining with hematoxylin followed by differentiation in HCl/ethanol, conditioning with the plant extract, staining with the red dye, differentiation in acetic acid/ethanol, and staining with the green dye.

With CellDetect, the nucleus of the malignant cell is stained in red. The cytoplasm of cancer cells is often stained in pink, especially when cells are arranged in clusters. Normal urothelial or squamous epithelial cells typically have a dark purple or green nucleus and greenish cytoplasm. Inflammatory cells are stained in purple/red, and are distinguishable based on their morphology.

All CellDetect stained smears were assessed under a light microscope (Olympus Life Science Solutions, Center Valley, PA, USA) by two independent cytologists. Five optional results were assigned: negative, reactive/inflammatory, suspicious, highly suspicious, and positive for UCC. These options were implemented based on a practice routinely used by local pathology departments when evaluating cytology slides. For study purposes, and based on an interim analysis, the three latter CellDetect outcomes were considered as a positive result. In case of discordance between cytologists, a third cytologist reviewed the slides to render a definitive diagnosis. Urine cytology was performed and analyzed in each of the medical centers according to institution-specific standard procedures. For the purpose of comparison, urine cytology was considered as positive when Grade III–IV of *atypia* was found.

2.3. Statistical analysis

Cases with negative cystoscopy or histology were considered negative while cases with positive histology were considered positive.

The sample size of the two cohorts was estimated based on the expected prevalence of UCC in each cohort and on point estimation of CellDetect performance to provide over 80% statistical power. Since the prevalence of UCC is estimated at around 10% in the routine cystoscopic surveillance cohort and 70% in the cohort of patients scheduled for TURBT, 40% of the specimens were obtained from the first cohort and 60% from the second cohort. Using simulations, the minimum sample size was estimated at 194 including 84 positive and 110 negative samples.

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Fig. 1 – Photomicrographs of urine smears stained with CellDetect. All parts are images of urine cytocentrifuge smears of a (A) normal individual or (B– H) urothelial carcinoma patients. Biopsy-confirmed stage and grade are indicated on the top of the corresponding images. Epithelial cells are stained in green. Images B–H show dysplastic cells exhibiting reddish-purple nuclei (digital zoom in the boxes). (H) Hemolysis is stained in green and inflammatory cells are purple (marked with arrows). Magnification: ×40, bar (presented on image H) = 50 µm. HG = high-grade; LG = low-grade.

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Sensitivity and specificity were tested by constructing a two-sided 95% confidence interval and demonstrating that their lower limit was above 0.70 and 0.65, respectively.

All statistical analyses were performed using SAS version 9.4 (SAS Institute Inc., Cary, NC, USA). Sensitivity, specificity, and the corresponding confidence intervals were estimated using the SAS GLIMMIX procedure (SAS Institute Inc., Cary, NC, USA).

3. Results

Two hundred and ninety one urine specimens obtained from patients monitored for UCC between April 2013 and

Enrollment

Urine specimens from patients with history of bladder cancer (n = 291) Cystoscopy TURBT Cystectomy (n = 148)(n = 125)(n = 18)Ineligible by adequacy assessment (n = 52)• Obscuring inflammation (15) Obscuring hematuria (4) Poorly preserved cells (12) Low cellularity (21) Ineligible by technical failure (n = 22)Analysis Eligible urine specimens (n = 217)Cystoscopy TURBT Cystectomy (n = 88)(n = 115)(n = 14)Normal cystoscopy (n = 88)Nonmalignant TURBT (n = 33) Positive for UCC (n = 96)NMIBC (n = 70)MIBC (n = 22)Others (n = 4)LGNMIBC **HGNMIBC**

Fig. 2 - Consolidated Standard of Reporting Trials flow diagram of patients and outcomes.

HGNMIBC = high-grade nonmuscle invasive bladder cancer; LGNMIBC = low-grade nonmuscle invasive bladder cancer; MIBC = muscle invasive bladder cancer; NMIBC = nonmuscle invasive bladder cancer; TURBT = transurethral resection; UCC = urothelial carcinoma.



October 2014 were stained with CellDetect. The enrollment of patients undergoing routine cystoscopy was discontinued once enough samples from this cohort were recruited according to sample size estimations. Recruitment of patients scheduled for TURBT or radical cystectomy continued until the prerequisite number of samples was obtained from this cohort as well. Fig. 2 illustrates the study design.

Technical exclusion of the samples was implemented according to the Bethesda guideline used for reporting Pap smear results. A total of 52 specimens (18%) were excluded based on adequacy assessment: obscuring inflammation

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(15), obscuring hematuria (4), poor cell preservation (12), and hypocellularity (21). Twenty-two samples were rejected due to technical failure during slide preparation.

Two hundred and seventeen urine specimens obtained from 208 patients (162 men and 46 women; aged 72 ± 10 yr) were included in the study. Nine samples were obtained from the same patients during follow-up visits since resampling was allowed by the study protocol if at least 3 mo separated the two visits. One hundred and thirty patients received intravesical washes, but no lesser then 4 wk prior to study enrollment. Eighty-six received Bacillus Calmette–Guérin, 33 mitomycin, five both Bacillus Calmette–Guérin and mitomycin, and six received synergo.

Concordance between the two cytologists was reported in 180/217 specimens (83%). The examination of a third observer was solicited in cases of discrepancy.

Patients' demographics and case distribution are shown in Table 1. Among the 217 cases, 96 were UCC-positive and 121 were proven negative by either normal cystoscopy (n = 88) or nonmalignant histological findings on biopsy (n = 33). The latter group included inflammation or reactive changes (n = 17), urothelial papilloma (n = 3), cystitis (n = 3), or other findings (n = 10).

Among the UCC-positive cases, 41 (43%) were low-grade nonmuscle invasive bladder cancer (LGNMIBC), 29 (30%) were high-grade NMIBC, 22 (23%) were muscle-invasive (MIBC), one was Tis, and in three cases a stage or grade was not assigned.

CellDetect accurately identified 81/96 positive cases, translating into 84% sensitivity. There was no age difference between CellDetect-positive and CellDetect-negative cases (72 ± 11 and 70 ± 11 , respectively). Tables 2 and 3 depict the findings in reference to the final diagnosis. Notably, a similar sensitivity was observed across all cancer grades and stages.

CellDetect correctly classified 85/121 UCC-negative cases, translating into 70% specificity. However, as this population was comprised of an unusually large proportion of patients undergoing TURBT for seemingly benign lesions (28%), this led to an over-representation of nonmalignant conditions. To overcome this over-representation, specificity was adjusted for the estimated rate of patients sent to the TURBT clinic following abnormal cystoscopy findings (10% compared with > 50% in the study population). This adjusted specificity was 83% and is comparable to the specificity of the stain in patients undergoing routine surveillance by cystoscopy (88 patients). Within this population, which truly represents the actual patients that will benefit from the test, the specificity of the stain was 84%. The sensitivity could not be calculated based on this subgroup owing to the low number of positive cases (n = 6).

Thirty-four patients with a positive CellDetect reading without evidence of UCC (false positives; FP) were followed for a median of 9 mo and their outcome was compared with healthy individuals classified as true negative. Seven out of 34 patients in the FP group (21%) developed a biopsy-proven UCC recurrence, whereas only three out of 65 true negative cases (5%) had disease relapse (p < 0.05). One patient in the FP group was subsequently diagnosed with upper tract UCC which may explain the positive cells found by CellDetect.

Table 1 – Patient demographics and case distribution

Criteria	No.		No.		
Gender					
Male			162		
Female			46		
Age			Yr		
Range			35-94		
Average \pm SD		72 ± 10			
Reference standard diag	nosis		No. o	f samples	
Negative cystoscopy			88		
Biopsy negative for UCC			33		
Biopsy positive for UCC			96		
Total			217		
Cancer grade/stage					
LGNMIBC			41		
HGNMIBC			29		
MIBC			22		
Tis			1		
Undetermined			3		
Total			96		
HGNMIBC = high-grade	nonmuscle	invasive	bladder	cancer:	

LGNMIBC = low-grade nonmuscle invasive bladder cancer; MIBC = muscle invasive bladder cancer; SD = standard deviation; UCC = urothelial carcinoma.

Table 2 – Accuracy parameters for CellDetect and comparator urine test

Parameter/test name	CellDetect	BTA stat	Cytology
Sensitivity (%)	84	69	50
Specificity overall (%)	70	70	87
Specificity cystoscopy-only group (%)	84	76	89

We compared the CellDetect results to those of BTA stat (215 cases) and urine cytology (172 cases). The sensitivity of CellDetect was superior to that of BTA stat and urine cytology (84%, 69%, and 50%, respectively, $p \le 0.05$; Table 2). The improved sensitivity of CellDetect was pronounced in both LGNMIBC (78% vs 54% and 33%, $p \le 0.05$; Table 3) and high-grade MIBC (93% vs 72% and 59%, $p \le 0.05$; Table 3). As displayed in Table 2, the specificity of BTA stat and urine cytology were 70% and 87%, respectively, in the overall population, and 76% and 89% in the cohort of patients undergoing routine surveillance by cystoscopy.

To test whether the CellDetect staining might be hampered by the presence of inflammation or hematuria, we used Multistix results to stratify cases as noninflammatory (leukocytes \leq 10/high power field [HPF]), inflammatory (leukocytes \geq +25/HPF), nonhematuria (red blood cells [RBC] \leq 15/HPF), and hematuria (RBC \geq +70/HPF) and tested the accuracy of the staining stratified by these subgroups. Both sensitivity and specificity were retained irrespective of the presence of inflammatory or RBC cells: 93% sensitivity and 96% specificity when applied to 53 samples with marked inflammation and 90% sensitivity and 90% specificity when applied to 29 samples with hematuria.

4. Discussion

In spite of the intensive efforts invested in enhancing existing therapy, the ability to treat NMIBC has not improved significantly overtime as 15% of the patients, particularly those at high risk tend to progress to higher stage associated

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Subgroup Test Total Eligible Positive Negative Sensitivity (%) LGNMIBC CellDetect 57 41 32 9 78 BTA stat 41 22 19 54 Cytology 30 10 20 33 HGNMIBC 29 93 CellDetect 29 27 2 BTA stat 29 21 7 72 17 10 7 59 Cvtology MIBC CellDetect 25 22 18 82 4 20 22 2 91 BTA stat 13 10 3 77 Cytology Tis CellDetect 1 0 1 1 BTA stat 1 1 0 0 Cytology 1 1 Undetermined CellDetect 6 3 3 0 BTA stat 3 2 Cytology 1 0 1

Table 3 – Assay sensitivity stratified by cancer grade/stage

HGNMIBC = high-grade nonmuscle invasive bladder cancer; LGNMIBC = low-grade nonmuscle invasive bladder cancer; MIBC = muscle invasive bladder cancer.

with a remarkable increase in mortality [20]. Thus, early diagnosis and timely eradication of the tumor remains a cornerstone in managing these patients successfully.

In this study, we expand our prior observation [19] and assess the performance of CellDetect in 217 urine samples on a blinded setting. Similar to prior results, the overall sensitivity remained high. Moreover, using a larger cohort, we were able to confirm that high diagnostic accuracy was maintained in recurrent LGNMIBC and demonstrated the method's relative advantage over available assays such as urine cytology and BTA stat. This is particularly promising in the context of recent findings showing limitations of urine markers to correctly diagnose recurrent tumors presumably because of their small size [21].

The overall study specificity, when including both cohorts of patients, was 70%. However, enrichment with patients scheduled for TURBT/radical cystectomy (> 50% of the study population) led to the over-representation of patients who had surgical intervention for apparently nonmalignant lesions. Since the intended use of the test is for patients undergoing routine outpatient surveillance, the more representative assay specificity is the one that refers solely to the 88 patients with negative cystoscopies (ie, 84%). This was further confirmed when adjusting the specificity of the overall study population for the estimated 10% rate of patients who are referred to the TURBT clinic while undergoing routine cystoscopic surveillance.

Of the 121 patients with no evidence of malignancy, 30% were classified as CellDetect-positive. Follow-up data showed that the recurrence rate among these patients was significantly higher compared to CellDetect-negative patients with no disease (21% vs 5%, p < 0.05). Whether CellDetect predates the overt diagnosis of bladder/upper tract relapse and can serve as an early indicator of recurrence to modify the follow-up intervals in this setting warrants further investigation.

Surveillance of NMIBC remains a daunting task for both patients and physicians as it entails frequent office visits and invasive procedures. Follow-up regimens have been a subject of ongoing research and multiple guidelines, reflecting the inconsistency in natural history spanning from indolent recurrence to life threatening disease progression. While rigorous cystoscopic surveillance remains imperative in monitoring of high-risk patients, the burden of invasive procedures may be diminished in low-risk patients, provided an adequately sensitive urinebased assay is available. A recent meta-analysis showed that available urinary biomarkers may miss a substantial proportion of patients with bladder cancer and are often subject to false-positive results. Accuracy is generally poor for low-stage and low-grade tumors [22].

There are several limitations to our study. First, the assay was performed in a central laboratory, thus additional work is underway to assess the test usability and staining reproducibility. Secondly, while CellDetect demonstrated superiority over available noninvasive urinary markers, the lack of urine cytology testing in some institutions, and the availability of additional assays that were not tested herein, limit this observation. Thirdly, the relatively small number of positive cases in an outpatient bladder surveillance cohort was overcome by enrichment with patients undergoing surgery for a tentative diagnosis of UCC. While statistically valid, this study design did not permit the estimation of the test performance in the target population of patients. Fourthly, patients with catheters, neobladder, ileal conduit, or kidney stones were excluded from the study and the effect of these interferences should be assessed in future investigation. Finally, 18% of the samples were found not eligible for evaluation according to the Bethesda guideline used for reporting cervical smear results. This proportion may reflect the lack of clear criteria for the adequacy of voided urine cytology [23]. These limitations warrant further investigation in order to evaluate the applicability and performance of the method in routine clinical practice.

5. Conclusions

This study validates the ability of CellDetect to accurately identify cancer cells in urine specimens of patients undergoing routine bladder cancer surveillance. The Cell-Detect assay retained its accuracy irrespective of tumor

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stage and grade, suggesting a putative role in both low- and high-risk disease. Further estimation of the test performance in the routine surveillance of patients with NMIBC is warranted.

Author contributions: Noa Davis had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: Davis.
Acquisition of data: Leibovitch, Nativ, Cohen, Mor, Lindner, Matzkin, Tsivian, Gofrit, Yossepovitch.
Analysis and interpretation of data: Shtabsky, Lew, Rona.
Drafting of the manuscript: Glickman, Davis.
Critical revision of the manuscript for important intellectual content: All authors.
Statistical analysis: Davis, Glickman.
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