A Novel Urine Cytology Stain for the Detection and Monitoring of Bladder Cancer

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Abbreviations and Acronyms

UC = urothelial carcinoma

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Purpose: CellDetect® is a unique platform technology comprising a proprietary plant extract and 3 dyes that enables color discrimination between malignant (red) and benign (green) cells based on specific metabolic alterations exclusive to the former. Preclinical studies and clinical trials demonstrated the applicability of the new technology in many cell culture lines and various cancers. We explored its performance characteristics in bladder cancer.

Materials and Methods: We performed an open label, 2-step study at tertiary medical centers. The study enrolled patients with newly diagnosed or a history of urothelial carcinoma. Step 1 involved staining archived biopsies. Slides were evaluated by 2 independent pathologists, who determined the concordance of the new staining technology with the hematoxylin and eosin based diagnosis. Step 2 included staining urine specimens with the new method and comparing findings to the patient final diagnosis and the results of standard urine cytology.

Results: A total of 58 archived biopsies were collected. The concordance of staining using the new platform technology with the hematoxylin and eosin based diagnosis was 100%. The new method applied to 44 urine smears showed 94% sensitivity and 89% specificity to detect urothelial carcinoma. Compared to standard urine cytology the new technology had overall superior sensitivity (94% vs 46%), particularly for low grade tumors (88% vs 17%, each $p < 0.005$). There was no significant difference in specificity between the 2 staining techniques.

Conclusions: Findings show the capability of CellDetect to accurately identify urothelial carcinoma. This indicates that the technology can be further developed to provide an alternative urine cytology test with diagnostic value that may have significant clinical benefits.

Key Words: urinary bladder, urothelium, carcinoma, diagnostic tests and procedures, staining and labeling

Bladder cancer is the fourth most common cancer in males and the eighth most common cause of cancer death with an estimated incidence of more than 74,690 new cases expected to have been diagnosed in 2014 in the United States and more than 15,000 deaths.[1] In patients diagnosed with nonmuscle invasive tumors there is up to an 80% chance of tumor recurrence,[2] rendering bladder cancer one of the most prevalent malignancies. Clinical guidelines recommend that patients with stage Ta, Tis or T1

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bladder cancer should be followed with cystoscopy every 3 months for the first 2 years after tumor resection, semiannually during the subsequent 2 years and annually thereafter.[3] However, actual surveillance of these patients often deviates from standard protocols,[4] mostly due to the heavy work burden imposed on physicians, and the associated pain and discomfort that discourage patients.

In the search for alternative noninvasive diagnostic tools numerous urinary biomarkers to detect bladder cancer were developed and commercialized in the last 2 decades.[5,6] To date none of these tools has been implemented in routine clinical practice to supplant cystoscopy as the standard of care.

CellDetect technology is a novel cell staining method based on a proprietary plant extract that enables color discrimination between benign and malignant cells while preserving critical features of cell morphology.[7–10] The discriminative capacity of the stain is related to specific metabolic alterations and increased metabolic activity observed in neoplastic cells. Preclinical studies and clinical trials demonstrated the applicability of this technology in many cell culture lines and various cancers.[8,10] We explored the performance characteristics of CellDetect technology in bladder cancer.

METHODS

We performed an open label 2-step study in accordance with the Good Clinical Practice, Declaration of Helsinki 2008, and the Ministry of Health requirements and regulations in Israel. Step 1 involved staining archived bladder tumor specimens using the new CellDetect technology. Step 2 included staining voided urine specimens with the new technology. Specimens were obtained from patients with an intact bladder undergoing routine cystoscopic followup after resection of nonmuscle invasive bladder cancer who were deemed free of disease 12 months or longer before study participation. These patients served as controls. Specimens were also obtained from patients diagnosed with bladder cancer by cystoscopy who were scheduled for transurethral resection of bladder tumor.

Urine samples were obtained before cystoscopy or tumor/bladder removal. The minimal volume of urine required for analysis was 50 ml. All samples were analyzed by microscopy using a Neubauer hemocytometer. Samples that contained high levels of obscuring elements, eg erythrocytes or leukocytes, and those that were oligocellular were considered technically inadequate and excluded from analysis.

Serial sections from archived biopsies were deparaffinized and rehydrated. One section per biopsy was stained with hematoxylin and eosin according to standard protocol. Adjacent tumor sections were stained by CellDetect according to manufacturer instructions. Briefly, the CellDetect kit contains 4 principal components, including a proprietary plant extract and 3 dyes. The staining protocol involves fixation with 10% trichloroacetic acid followed by nuclear staining with hematoxylin and serial incubations in kit proprietary components with intermittent washes. All sections stained by this new method were analyzed by 2 independent pathologists using white light microscopy. The diagnosis was based on cell color and morphology. These readings were compared to the diagnosis based on hematoxylin and eosin staining, and the concordance between the 2 methods was assessed.

Urine samples were collected in clinic during the morning hours but not as first morning urine. They were processed to cytospin smears and fixed with 96% ethanol. Smears (1 slide per patient) were stained by the new method and analyzed by an expert cytopathologist under 20× magnification. Cell morphology was determined according to standard cytological criteria, namely an increased nucleus-to-cytoplasm ratio, nuclear irregularity, nuclear polymorphism and nucleoli. Cytoplasm and nucleus color was also documented. Cytology readings using the new method were compared to the patient final diagnosis and standard urine cytology readings based on hospital records when available.

RESULTS

A total of 58 eligible archived biopsy specimens were retrieved, including 22 (38%) with normal mucosa, 17 (29%) with stages Ta, T1 and Tis bladder cancer, and 19 (33%) with muscle invasive tumor (stage T2 or greater). Figure 1 shows representative images of CellDetect staining technology applied to tissue specimens. Cells comprising normal transitional

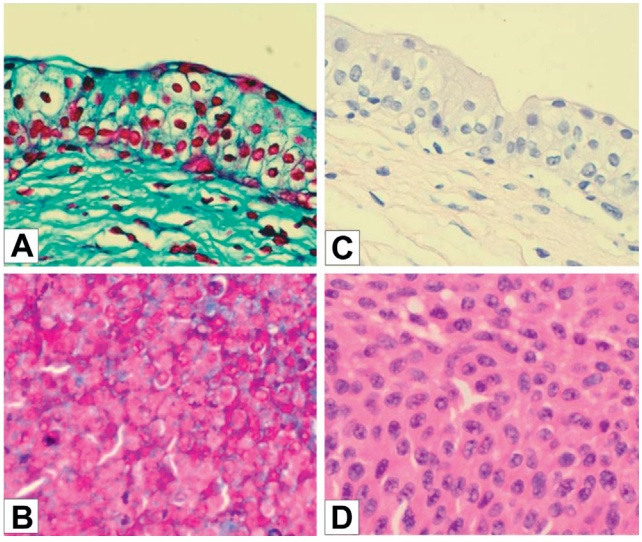

Figure 1. Photomicrographs show bladder transitional epithelium biopsy histological sections of normal epithelium (*A* and *C*) and UC (*B* and *D*). Cytoplasmic green/blue staining is characteristic of nonneoplastic states while neoplasm consists of cells with pink-magenta stained cytoplasm. CellDetect staining (*A* and *B*) and H&E (*C* and *D*), reduced from ×40.

epithelium had a greenish-blue cytoplasm (fig. 1, *A* and *C*). Morphologically recognizable neoplastic cells showed a red/magenta tinged cytoplasm (fig. 1, *B* and *D*). Based exclusively on the tinctorial status of the epithelium it was possible to clearly distinguish neoplasm from normal epithelium even at low magnification. Concordance between the diagnoses made by the new staining method and that established by hematoxylin and eosin staining was 100%.

Of 148 collected urine samples 69 were used for staining calibration (with staining protocol adaptation from tissue samples to cell smears), 30 were considered technically inadequate due to severe inflammation, low cellularity or poor cell preservation and 5 were excluded from analysis due to the lack of a subsequent confirmatory biopsy. Thus, 44 samples were available to analyze the performance characteristics of the new staining technology in urine. Multiple slides were prepared from urine collected from each patient. Each was stained independently by the same technician or by 2 operators. We found minimal variation in cell color and nonspecific background, of which none altered the final reading or diagnosis.

The study included 27 healthy individuals and 17 patients with cancer (table 1). In general the new staining technology discriminated neoplastic cells, which stained red, from surrounding normal cells, which stained green (fig. 2). The red dye highlighted the nucleus and often the cytoplasm of neoplastic/dysplastic cells while the target of the green dye was the cytoplasm of normal cells (fig. 2). Cytomorphology features consistent with neoplasm/dysplasia were not altered by the staining technique and assisted in confirming the diagnosis. The nucleus of cells with reactive changes stained green to purple (fig. 2, *B*). The nucleus-to-cytoplasm ratio was significantly less, enabling discrimination from dysplastic/neoplastic cells. Other cells detected on slides were erythrocytes and leukocytes, which were easily identified by morphology and size.

To evaluate the association between cellular morphology and the color readings of the new staining method we studied the morphological features of a total of 1,031 cells from 14 patients with cancer and 8 healthy controls. Using the new method positive staining was defined as a red/purple nucleus on a background of pink or green cytoplasm while negative staining was defined as a green/blue nucleus and a greenish cytoplasm. Of 389 cells 362 (93%) showed morphological features consistent with malignancy and were categorized as positive staining. Of 690 normal cells 669 (97%) were categorized as negative staining (table 2).

We then compared CellDetect urine staining results and the final pathological diagnosis established by cystoscopy in healthy individuals with hematoxylin and eosin staining results in biopsy specimens from patients who underwent

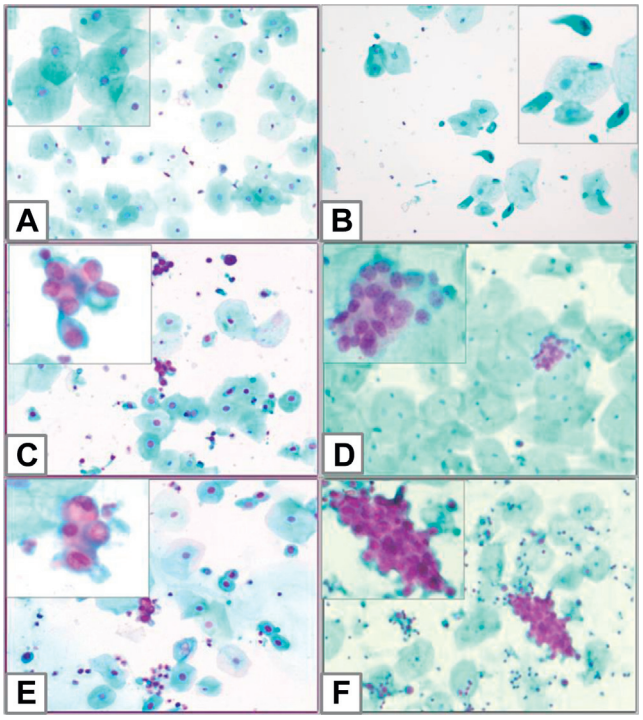

Figure 2. Photomicrographs show urine smears stained with CellDetect, including urine cytospin smears of normal subject (*A* and *B*) and patients with UC (*C* to *F*). Most cells were normal and epithelial cells stained green. Note reactive cells, which stained green (*B*), and few foci of reddish cancer cells (*C* to *F*). Cancer cells preserved distinctive morphology with enlarged, polymorphic nucleus and little cytoplasm. Reduced from ×40.

Table 1. *Clinical diagnosis groups*

	No. Pts
Diagnosis:	44
No disease evidence	27
Ca	17
Ca grade:	17
Low	8
High	9
Ca stage:	17
Ta	10
Tis	1
T1	5
T3	1

Table 2. *CellDetect stain color/morphology correlation*

Stained Cells	No. Cells (%)
Ca:	
Pos	362 (93)
Neg	27 (7)
Normal:	
Pos	21 (3)
Neg	669 (97)

transurethral resection of bladder tumor. We analyzed 44 eligible urine samples, including 27 from normal subjects and 17 from patients with recurrent (15) or primary UC. All normal subjects were deemed tumor free 1 year or longer before study enrollment. Of 27 normal subjects 19 received instillation, including intravesical bacillus Calmette-Guérin in 7, intravesical mitomycin C in 8 and a combination of the 2 instillations in 4. The new technology accurately identified 16 of 17 cancer cases and 24 of 27 healthy controls. Thus, it had 94% sensitivity, 89% specificity, 84% negative predictive value and 96% positive predictive value (table 3). Notably the new staining method accurately identified 7 of 8 patients with low grade noninvasive tumors and all 9 with high grade UC.

We further compared the performance of the new technology with that of urine cytology based on hospital records of study participants. A total of 34 cytology records were available for this analysis. Compared to standard urine cytology the new staining technology demonstrated overall superior sensitivity (94% vs 46%, $p < 0.005$, table 3), particularly in low grade tumors (88% vs 17%, $p < 0.005$, data not shown). There was no significant difference in specificity between CellDetect and the urine cytology technique (89% and 95%, respectively, $p = 0.2$, data not shown).

DISCUSSION

The high prevalence of bladder cancer and the requirement for frequent long-term bladder surveillance renders bladder cancer the most expensive malignancy[11] with an estimated lifetime cost per patient as high as $200,000.[12] Thus, an effective, low cost, noninvasive urine based assay to detect bladder cancer would be highly valuable for patients and the health care system.

To our knowledge this study represents the first application of CellDetect technology in urological oncology. This novel staining technology discriminated benign from malignant transitional epithelium fairly accurately. Nuclear staining of neoplastic cells was consistently red to purple, clearly distinguishable from the green shade of nonneoplastic (normal and reactive) cells. Also, applying the staining procedure to urine smears and histological specimens was straightforward and reproducible. However, because there was no apparent advantage to the CellDetect method over traditional hematoxylin and eosin staining in tissue biopsy specimens, the added benefit and future application of this technology is likely to be in voided urine specimens.

Table 3. *CellDetect and urine cytology performance at all cancer stages*

	CellDetect	Urine Cytology
No. pts	44	34
% Sensitivity	94	42
% Specificity	89	95
% Predictive value:		
Neg	84	83
Pos	96	75

The new method correctly identified 16 of 17 cancer cases in voided urine specimens, specifically 7 of 8 cases of low grade disease. An acknowledged limitation of urine cytology, particularly in the low grade setting, is its low sensitivity, which has been a major hurdle in routine bladder cancer surveillance.[13] Thus, the high sensitivity of the new staining method in this study may enable patients to forgo some required invasive cystoscopies.

Notably this high sensitivity did not come at the expense of compromised specificity since 24 of 27 normal subjects were diagnosed accurately by the new method. It remains to be determined whether bladder tumors develop in the near future in the 3 cases classified as false positive.

Taken together we believe that CellDetect represents a promising novel noninvasive staining technology that serves as an adjunct to cystoscopy. However, these findings are based on a limited number of cases and need further validation in a large prospective study.

What are the biological grounds for CellDetect staining? Neoplastic cells show striking alternations in metabolism, manifesting as a shift in glucose metabolism from oxidative phosphorylation to glycolysis and culminating in intracellular alkalosis. The new technology is assumed to rely on this phenomenon by capturing the unique metabolic signature resulting from the shift in energy metabolism. Based on differential pH affinity the combination of dyes enables color discrimination, which is accentuated and stabilized by the proprietary plant extract. This differential metabolic activity of malignant cells leads to prominent changes in intracellular proteins, which are preserved in fixated cells and in turn react differently with the dyes upon exposure to the plant extract. Further research is now being done to explore the exact molecular mechanism underlying this phenomenon.

A major limitation of this study is its small sample size, primarily due to exclusion of a large number of technically inadequate samples resulting from hypocellularity or the presence of inflammatory cells. Urine is generally considered to be oligocellular and slides containing few to no cells are common when assessing urine cytology smears.[14] However, unlike in other cytology tests such as cervical cytology there are no consensus guidelines regarding the minimal number of cells that define a

sample as adequate. Further study is warranted to determine the minimal number of cells needed for reliable CellDetect analysis.

Inflammation is a known cause of false-positive findings in bladder cancer diagnostics.[15] In many studies in the field it was considered an exclusion criterion to improve assay specificity.[16] Because this study is our initial proof of concept meant to calibrate the staining protocol, we refrained from including suboptimal samples, such as those that were oligocellular or had a large amount of inflammatory cells, since this may have introduced bias. Current studies are being performed to overcome these hurdles, including liquid based cytology to filter inflammatory cells and increasing urine volume to collect more cells.

CONCLUSIONS

These findings demonstrate the ability of the CellDetect technology to accurately identify UC in biopsy and voided urine specimens. This technology may provide an alternative to standard urine cytology with significant clinical benefit.

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