Diagnosis of Genito-Urinary Tract Cancer by Detection of Minichromosome Maintenance 5 Protein in Urine Sediments

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Background: **Because cystoscopy is invasive and expensive and urine cytology has low sensitivity, alternative methods for detecting bladder cancer are sought. Minichromosome maintenance (Mcm) proteins have been used as diagnostic markers for cervical cancer. We investigated whether one Mcm protein, Mcm5, can be used to detect urothelial cancer cells in urine sediments.** ***Methods:*** **We used two monoclonal antibodies against His-tagged human Mcm5 (amino acids 367–582) in an immunofluorometric assay to measure Mcm5 levels in cells in the urine of 353 patients who presented with hematuria or lower urinary tract symptoms or who were undergoing follow-up cystoscopy for urothelial neoplasia. Urine samples were also subjected to routine cytologic analysis. Patients underwent upper urinary tract imaging and cystoscopy within 12 hours of producing the urine sample. Data were analyzed by comparing areas under a nonparametric receiver operating characteristics (ROC) curve and by McNemar's test and Fisher's exact test. All statistical tests were two-sided.** ***Results:*** **At the assay cut point where the false-negative and false-positive rates were the same, the Mcm5 test detected primary and recurrent bladder cancers with 87% (95% confidence interval [CI] = 77% to 94%) sensitivity and 87% (95% CI = 83% to 91%) specificity. At the cut point where the specificities of urine cytology and the Mcm5 test were equal (97%, 95% CI = 95% to 99%), the Mcm5 test was statistically significantly (*P*<.001) more sensitive than urine cytology, 73% (95% CI = 61% to 83%) versus 48% (95% CI = 35% to 60%). At the lower detection limit of the Mcm5 test, sensitivity was highest, 92% (95% CI = 83% to 97%) and specificity was 78% (95% CI = 72% to 83%). Patients with prostate cancer had higher levels of Mcm5 in their urine sediments than did men without malignancy (*P*<.001).** ***Conclusions:*** **Elevated levels of Mcm5 in urine sediments are highly predictive of bladder cancer. [J Natl Cancer Inst 2002;94:1071–9]**

Bladder cancer is a common disease in the United States: It has an annual incidence of over 50 000 and causes more than 12 500 deaths per year. It is the fourth most common cancer in men and the sixth most common cancer in women. Worldwide, an estimated 243 000 cases of bladder cancer occur each year; incidence rates are highest in industrialized countries, where over 90% of bladder cancers are of transitional cell origin *(1).* Approximately 75% of patients initially diagnosed with transitional cell carcinoma present with superficial tumors that can be treated by transurethral resection and intravesical therapy alone. Another 20% of these patients have invasive transitional cell carcinoma, and the remaining 5% present with metastatic tumors *(2).* Clinical management of patients with transitional cell carcinoma is complicated because the recurrence rate of superficial disease is greater than 60%, and the disease progresses to a higher stage or grade in 42% of patients within 10 years of diagnosis *(3).* Thus, early detection and monitoring of patients with bladder cancer may be important for successful treatment.

Cystoscopy is the gold standard diagnostic test for bladder cancer because it allows direct visualization and biopsy of the bladder urothelium. Cystoscopy is routinely used to test patients that present with painless hematuria or irritative voiding, both symptoms of early transitional cell carcinoma that are more often related to less serious diseases, such as urinary tract infection or benign prostatic hyperplasia. Patients with these nonspecific symptoms may undergo extensive urological investigations, even though only a small percentage of them actually have malignancies *(4).* Because cystoscopy is invasive and costly, both patients and clinicians would benefit greatly from the development of cost-effective and noninvasive tools for the diagnosis and surveillance of bladder cancer.

Cytologic analysis of voided urine is the most commonly used noninvasive method for detecting transitional cell carcinoma, but its utility is severely constrained by its low sensitivity, which is only 20%–40% for low-grade tumors *(5).* Recently, however, several potential diagnostic markers for bladder cancer have been identified, including nuclear matrix protein 22, bladder tumor antigen, and telomerase. Although these markers are more sensitive than urine cytology for detecting bladder cancer, their use is limited by low specificity *(6).*

Specific genetic alterations have been implicated in the molecular pathogenesis of transitional cell carcinoma, with mutations reported in cell cycle regulatory genes, oncogenes, and tumor suppressor genes *(7–13).* However, it has proven difficult to use these genetic alterations as diagnostic markers of bladder cancer because of their low sensitivity. Ras oncogenes, for example, are expressed in only 10%–36% of bladder cancers *(14,15),* and p53 mutations are present in only 60% of advanced

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See "Notes" following "References."

invasive transitional cell carcinomas *(8).* Similarly, adequate sensitivity using microsatellite analysis can be achieved only by targeting up to 20 different loci, making the technique complex and expensive *(16).* More important, targeting multiple genetic loci increases the probability of missing early transitional cell carcinoma because these tumors harbor fewer genetic lesions.

The initiation of DNA replication represents a potentially attractive target for diagnostic analysis *(17).* Proteins of the minichromosome maintenance (Mcm) family (Mcm2, Mcm3, Mcm4, Mcm5, Mcm6, and Mcm7), originally identified in yeast in a genetic screen for mutants defective in the extrachromosomal replication of minichromosomes *(18),* play a critical regulatory role in the initiation of DNA replication *(19).* We have previously demonstrated that deregulated expression of MCM proteins is characteristic of epithelial cell carcinogenesis, resulting in the exfoliation of MCM-positive tumor cells *(20),* and have used these novel markers of growth in diagnostic screening applications for cervical cancer *(21).* Because MCM proteins identify both proliferating cells and nonproliferating cells with proliferative potential, MCM protein expression is a more sensitive marker of epithelial carcinogenesis than is expression of conventional proliferation markers *(22).* For example, the percentage of Mcm5-expressing cells in urothelial carcinoma is associated with the pathological grade of transitional cell carcinoma, averaging 78% for poorly differentiated G3 tumors, 70% for moderately differentiated G2 tumors, and 45% for well differentiated G1 tumors *(23).* These percentages correspond to a higher growth fraction than that identified by the conventional proliferation marker Ki67 (e.g., 16% for G3 tumors, 6% for G2 tumors, and 5% for G1 tumors) *(24).* On the basis of these data, we have previously suggested that the detection of MCM proteins in exfoliated cells in urine sediments is a potentially sensitive indicator of urothelial malignancy *(23).* Here we describe a critical evaluation of this approach using a liquid-phase immunofluorometric assay to quantitatively measure Mcm5 levels in the urine sediments of 353 patients with nonspecific lower urinary tract symptoms.

Materials and Methods

Study Subjects

Single voided urine specimens were obtained from 353 patients who presented with hematuria or lower urinary tract symptoms (e.g., frequent voiding, dysuria) or who were undergoing a follow-up cystoscopy for urothelial neoplasia at Addenbrooke's Hospital National Health Service Trust (Cambridge, U.K.). All patients underwent upper urinary tract imaging and cystoscopy within 12 hours of producing the urine sample. Male patients were examined by digital rectal examination and ultrasound for the presence of prostatic disease. For those suspected of having prostate cancer, prostate-specific antigen (PSA) levels were measured and needle biopsies of the prostate were obtained. Urine samples were analyzed in a blinded fashion for immunofluorometric Mcm5 detection and cytologic analyses. On completion of the study, we decoded the patient data and compared the immunofluorometric signals with results obtained from cystoscopy, biopsy histology, and urine cytology. Staging and grading of malignant tumors was performed using the TNM (tumor–node–metastasis) classification system *(25).* Ethical approval was obtained from the Local Research Ethics Committee.

Processing of Urine Sediments

Urine sediments were obtained by centrifuging urine samples at 1500*g* for 7 minutes at 4 °C. The pelleted material was resuspended in storage buffer (phosphate-buffered saline [PBS], 5% bovine serum albumin [BSA], 1 *M* sucrose, 0.02% NaN_3) that contained one complete EDTA-free protease inhibitor cocktail tablet (Roche Diagnostics Ltd., Lewes, East Sussex, U.K.) per 50 mL of buffer. The resuspended urine sediments were stored in liquid nitrogen within 2 hours of the urine samples being passed.

Urine Cytology

Urine samples (50 mL) were centrifuged at 1500*g* for 5 minutes. Cytospin preparations were prepared on poly-L-lysine-coated slides using Shandon cytospin tubes and cytocentrifuge according to the manufacturer's instructions (Thermoshandon Ltd., Runcorn, Cheshire, U.K.). Samples were fixed in industrial methylated spirits (BDH Laboratory Supplies, Poole, U.K.) and stained using the standard Papanicolaou technique for smears *(26).*

Preparation of Standards for Immunofluorometric Mcm5 Assay

HeLa S3 cells were cultured as exponentially growing monolayers in Dulbecco's modified Eagle medium (GIBCO BRL, Life Technologies Ltd., Paisley, U.K.) supplemented with 10% fetal calf serum (GIBCO BRL), 100 U/mL penicillin, and 0.1 mg/mL streptomycin in a 37 °C humidified incubator in the presence of 5% CO_2. HeLa cells were harvested after trypsinization and diluted with storage buffer to concentrations of 1500, 5000, 15 000, 50 000, and 150 000 cells per well. The zero-cell standard consisted of 500 μL of storage buffer. The standards were stored in liquid nitrogen and later used to generate a standard curve for Mcm5.

Processing of Standards and Clinical Samples

Standards and clinical samples were thawed, and the cells were isolated by centrifugation at 1500*g* for 5 minutes at 4 °C. The supernatants were discarded, and the cell pellets were washed three times with 500 μL of PBS. Cell pellets were resuspended in 250 μL (for those pellets with a volume less than approximately 200 μL) or 500 μL (for those pellets with a volume greater than approximately 200 μL) of processing buffer (PBS, 0.4% sodium dodecyl sulfate [SDS], 0.02% NaN_3). Cell lysates were prepared by incubating the resuspended samples at 95 °C for 45 minutes. The DNA in each sample was sheared by passing the lysates through a 21-gauge needle (Becton Dickinson UK Ltd., Cowley, Oxford, U.K.), and nucleic acids were digested with DNase I (20 U/mL; Roche Diagnostics) and RNase A (1 μg/mL; Roche Diagnostics) for 2 hours at 37 °C. The samples were centrifuged at 15 000*g* for 10 minutes to pellet the cell debris, the supernatants were collected, and 50 μL of each was directly used in the immunofluorometric assay.

Expression and Purification of His-Tagged Mcm5

Escherichia coli strain BL21 was transformed with plasmid pQEhsMcm5, which contains amino acids 367–582 of human Mcm5 fused to a histidine affinity tag under control of the phage T5 promoter with a double lac operator repression module (pQE70; Qiagen Ltd., Crawley, West Sussex, U.K.). An over-

night culture was grown at 37 °C with vigorous shaking in 20 mL of LB medium supplemented with 100 μg/mL ampicillin and 25 μg/mL kanamycin. The overnight culture was used to inoculate four flasks, each containing 500 mL of fresh LB medium. Cultures were grown at 37 °C with vigorous shaking until an optical density at 600 nm (OD_{600}) of 0.6–0.8 was reached. Isopropyl β-D-thiogalactoside was added to a final concentration of 2 m*M* to each flask, and the cultures were allowed to grow for an additional 5 hours at 37 °C. The cultures were chilled on ice, and cells were harvested by centrifugation at 4000*g* for 20 minutes at 4 °C. The resulting cell pellet was washed with PBS, the wet weight of the cells was determined, and the pellet was frozen at –80 °C.

Bacterial cell pellets were thawed, resuspended in 5 mL of lysis buffer (6 *M* guanidine–HCl, 0.1 *M* NaH_2PO_4, 0.01 *M* Tris–HCl [pH 8.0], 1 *M* NaCl, 0.1% Triton X-100, 20 m*M* 2-mercaptoethanol, 1 m*M* phenylmethylsulfonyl fluoride [PMSF]) per gram of wet cell weight, sonicated three times for 15 seconds, and stirred for 90 minutes at room temperature. The lysate was centrifuged at 100 000*g* for 1 hour in a Beckman Ti66 rotor to pellet cellular debris. The supernatant (cleared lysate) was transferred to a fresh tube and bound in a batch mode to 1 mL of Ni^{2+}-nitrilotriacetic acid (Ni-NTA) agarose (Qiagen), which had been pre-equilibrated in lysis buffer at room temperature for 1 hour with gentle shaking (200 rpm on a rotary shaker). The lysate–resin mixture was loaded into a column, and the resin was washed first with lysis buffer and then with wash buffer (8 *M* urea, 0.01 *M* Tris–HCl [pH 6.3], 1 *M* NaCl, 0.1% Triton X-100, 20 m*M* 2-mercaptoethanol, 1 m*M* PMSF). Bound protein was eluted in steps with elution buffer (8 *M* urea, 0.01 *M* Tris–HCl [pH 4.5], 1 *M* NaCl, 0.1% Triton X-100, 20 m*M* 2-mercaptoethanol, 1 m*M* PMSF). Protein-containing fractions were identified by dot blot, pooled, and concentrated using Vivaspin concentrators (VivaScience Ltd., Satorius Ltd., Epsom, U.K.), and then dialyzed against PBS 0.3% SDS.

Monoclonal Antibody Production and Purification

Monoclonal antibodies against bacterially expressed His-tagged human Mcm5 (amino acids 367–582) were raised in BALB/c×CBA F_1 mice. Mice approximately 3 months old were immunized by subcutaneous injection of 100 μg of bacterially expressed fusion protein that was emulsified with an equal volume of Titremax (Stratech Scientific Ltd., Luton, U.K.). The immunization was repeated three times at 2- to 3-month intervals, after which the mice were rested for 6 months. Another 100 μg of fusion protein in PBS 0.3% SDS was injected intraperitoneally at 6 days and 3 days before death. Hybridoma cell lines were established by fusing splenocytes from the immunized animals with the myeloma line Sp2/0-Ag14 by polyethylene glycol treatment *(27).* Hybridoma cell lines were screened for antibodies to the fusion protein by a dot blot procedure, and cell lines that expressed such antibodies were cloned at least three times before they were used for antibody production *(27).*

Hybridoma supernatants were passed through a protein A-Sepharose 4B Fast Flow bead column (30 mg immunoglobulin G [IgG]/mL binding capacity; Sigma-Aldrich Co. Ltd., Gillingham, U.K.) that was pre-equilibrated in PBS. The column was washed with PBS, and bound antibody was eluted slowly in steps with 100 m*M* glycine–HCl (pH 2.9, 1 mL/step) into tubes containing 1 *M* Tris (pH 9.6). The tubes were mixed gently to bring the pH values back up to 7.0–7.8. The absorbance at 280 nm was measured for each fraction, and antibody-containing fractions were pooled.

Protein A-purified monoclonal antibodies were screened against recombinant Mcm5 that was immobilized on Ni-NTA HisSorb™ microtiter plates (Qiagen). Bound monoclonal antibodies were detected by colorimetry at an absorbance of 450 nm after incubation with horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Dako, Ely, U.K.). Candidate monoclonal antibodies were tested in paired combinations for epitope specificity under competitive conditions in the Ni-NTA HisSorb™ screening assay according to the manufacturer's instructions, and two high-affinity monoclonal antibodies (MAbs; IgG2b isotype) were selected. These MAbs, named 12A7 and 4B4, were directed against different epitopes of Mcm5 and were used in a two-site time-resolved immunofluorometric assay to determine Mcm5 levels in urine sediments.

Dissociation-Enhanced Lanthanide Fluorometric Immunoassay (DELFIA) to Measure Mcm5 Levels

We labeled protein A-purified MAb 4B4 with europium *(28)* using a DELFIA® Eu-labeling kit (Perkin Elmer Life Science, Wallac Oy, Turku, Finland) according to the manufacturer's instructions. Europium-labeled MAb 4B4 (seven molecules of Eu per IgG) was stabilized with 5% (wt/vol) highly purified BSA and stored at 4 °C in 0.1 *M* bicarbonate buffer (pH 9.2) containing 0.02% NaN_3. The assay was standardized using HeLa cells, and one fluorescence unit was defined as the signal generated by the Mcm5 contents of one proliferating HeLa S3 cell, approximately 10^5 Mcm5 molecules *(29).* DELFIA® research reagents were obtained from Perkin Elmer Life Science. All other reagents were obtained from Sigma-Aldrich.

Nunc Maxisorp microtiter plate wells (Life Technologies) were coated by passive adsorption with 200 μL of 8 μg/mL protein A purified-MAb 12A7 and incubated overnight at room temperature. The microtiter wells were washed four times with DELFIA® wash buffer. Residual adsorptive sites on the microtiter plate wells were blocked with 300 μL of blocking buffer (PBS, 5% BSA, 0.02% NaN_3). After incubation at room temperature for 1 hour, the microtiter plates were washed three times with DELFIA® wash buffer, and then DELFIA® multibuffer (150 μL/well) was added. Duplicate wells containing 50 μL per well of blank in processing buffer (zero-cell standards), standards (HeLa S3 cells at 0, 1500, 5000, 15 000, 50 000, and 150 000 cells/well in processing buffer), quality controls (HeLa S3 cells spiked with acellular urine), and clinical samples in processing buffer were prepared, the wells were sealed with an adhesive plastic sheet to prevent evaporation, and the microtiter plates were incubated on a plate-shaker overnight at room temperature. Microtiter wells were washed six times with DELFIA® wash buffer, and 200 μL of europium-conjugated MAb 4B4 at 1 μg/mL was added. After a 4-hour incubation on a plate-shaker at room temperature, the microtiter plates were washed six times with DELFIA® wash buffer and then 200 μL of DELFIA® enhancement solution was added to each well. After 5-minute incubation on a plate shaker, the fluorescence emission (at 613 nm) of each well was read on a Wallac DELFIA® 1232 time-resolved fluorometer. Standard curves were constructed from fluorescence values generated by the blank and standard wells, and the fluorescence values of the clinical samples were calculated with the Multicalc Advanced Immunoassay Data Management package (Perkin Elmer Life Science).

Immunoassay Performance

A standard curve was produced with different numbers of HeLa S3 cells (fluorescence values were plotted against cell number), and the lower detection limit of the assay was determined by averaging the nonspecific binding signals of 19 zero-cell standards. Three standard deviations of the 19 measurements were added to the mean nonspecific binding signal, and the number of cells per well of the corresponding fluorescence signal was determined from the standard curve. These data showed that the assay could detect Mcm5 from as few as 809 HeLa cells per well. In our analysis of patient samples, we used 1500 cells/well as the lower detection limit because the within-batch variation of the assay for cell dilutions below 1500 cells/well was greater than 25%. Samples that generated a fluorescence signal below that corresponding to 1500 cells/well were reported as having fewer than 1500 cells/well.

Statistical Analysis

Sensitivity and specificity characteristics of the immunofluorometric Mcm5 test for the detection of bladder cancer are presented as a receiver operating characteristics (ROC) curve. The area under the nonparametric ROC curve was used to assess the overall accuracy of the test *(30).* Three cut points were used to demonstrate test performance under a range of circumstances: at the lower detection limit of the assay (i.e., 1500 cells/well), where sensitivity of the test was maximal; at the point where the false-positive and false-negative rates of the test were equal (i.e., 2500 cells/well); and at the point where the specificity of the test was 99% (i.e., 6000 cells/well). An exact 95% confidence interval (CI) for each proportion, including sensitivity, specificity, and predictive values of Mcm5 and cytology, was derived assuming a binomial distribution using StatXact software, version 4.0 (Cytel Software Corporation, Cambridge, MA). Unless otherwise stated, statistical tests were performed using SPSS software, version 9.0 (SPSS Inc., Chicago, IL). The sensitivity determined for urine cytology was compared with that of the immunofluorometric Mcm5 test using McNemar's test for paired proportions. The performance of the combination of the cytology and the immunofluorometric Mcm5 tests was assessed using the Multi-ROC method programmed in S-plus software (MathSoft Inc., Seattle, WA) *(31).* Fisher's exact test was used to compare sensitivity, specificity, and false-positive rates between distinct patient subgroups. False-negative rates were initially tested for evidence of trend using the linear-by-linear association chi-square test *(32)* with the "exact" option selected in SPSS and were then subsequently tested using Fisher's exact test to detect nontrend effects. The areas under the ROC curves were compared between distinct subgroups using the unpaired *t* test based on the estimated areas and their standard errors *(33).* The level of the signal was compared between patient groups using the Mann–Whitney *U* test and by using confidence intervals for differences between medians. All statistical tests were two-tailed, and a 5% level was used to indicate statistical significance.

RESULTS

The demographic characteristics, clinical symptoms on presentation, cystoscopy findings, and histopathological diagnoses of the 353 patients included in this study are summarized in Table 1. The study population was predominantly male (77%), had a median age of 70 years (interquartile range, 62–78 years), and presented for the first time with hematuria (82%). Forty-three patients (12%) had a previous history of transitional cell carcinoma, and 71 patients (20%) were found to have bladder tumors on cystoscopy. Most of the tumors were transitional cell carcinomas, which were evenly distributed across tumor grades; 30 (42%) were stage pTa, and 21 (30%) were stage pT1 *(25).* The urothelial neoplasms included seven cases (10%) of carcinoma *in situ.* Four of those tumors were of nontransitional cell origin; these were a B-cell non-Hodgkin's lymphoma, an extramedullary myelomonocytic leukemia, a sigmoid colonic adenocarcinoma with direct invasion of the bladder, and a primary small-cell neuroendocrine carcinoma of the bladder. The other three patients were diagnosed with renal transitional cell carcinoma with parenchymal invasion of the pelvis (two patients had G2pT1 tumors and one had a G3pT1 tumor). In addition, seven (3%) of the 273 male patients were found to have prostate cancer on presentation but no evidence of transitional cell carcinoma. These patients were omitted from the primary analyses shown in Tables 2, 3, and 4 but were included in the analysis presented in Table 5, where follow-up information has been updated and previously excluded groups are included.

Table 1. Patient demographics and tumor characteristics at cystoscopy

Characteristic	N (%) or median (interquartile range)
Sex	
Male	273 (77%)
Female	80 (23%)
Age, y	70 (62–78)
Presenting symptoms	
Hematuria	289 (82%)
Lower urinary tract symptoms	64 (18%)
Type of cystoscopy	
Primary	310 (88%)
Surveillance	43 (12%)
Median urine volume, mL	56 (43–85)
Tumor	
Absent	275 (78%)
Prostate cancer	7 (2%)
Bladder cancer*	71 (20%)
Grade†	
1	17 (24%)
2	23 (32%)
3	21 (30%)
Stage†	
pTa	30 (42%)
pT1	21 (30%)
pT2/pT3	10 (14%)

*Three renal transitional cell carcinomas were included in the analysis (two were G2pT1, one was G3pT1).

†Eleven (15%) of the 71 bladder tumors had no grade or stage information: one was a B-cell non-Hodgkin's lymphoma, one was an extramedullary myelomonocytic leukemia, one was a sigmoid adenocarcinoma, one was a small-cell neuroendocrine carcinoma, six were carcinomas *in situ* (CIS), and one was a CIS with coexisting G1pTa transitional cell carcinoma.

The performance of the immunofluorometric Mcm5 assay as a diagnostic test for bladder cancer is shown as an ROC curve (Fig. 1). The test discriminated, with high specificity and sensitivity, between patients with and without bladder cancer, as demonstrated by the large area under the ROC curve (0.93 [95% CI = 0.89 to 0.97]), which was statistically significantly larger than the area assumed by the null hypothesis (0.5) ($P<.001$). In other words, a randomly selected patient with bladder cancer would have a 93% probability of having an immunofluorometric

Table 2. Sensitivity, specificity, and predictive values of the Mcm5 test and urine cytology in all patients and in surveillance cystoscopy patients*

Test	% Sensitivity (95% CI)	% Specificity (95% CI)	% PPV (95% CI)	% NPV (95% CI)
		All patients (n = 346)†		
Mcm5 test				
⩾1500 cut point	92 (83 to 97)	78 (72 to 83)	52 (43 to 61)	97 (94 to 99)
⩾2500 cut point	87 (77 to 94)	87 (83 to 91)	64 (54 to 73)	96 (93 to 98)
⩾6000 cut point	72 (60 to 82)	99 (97 to 100)	96 (87 to 100)	93 (90 to 96)
Urine cytology positive‡	48 (35 to 60)	97 (95 to 99)	82 (66 to 92)	88 (84 to 92)
		Surveillance cystoscopy patients (n = 43)		
Mcm5 test				
⩾1500 cut point	92 (64 to 100)	80 (61 to 92)	67 (41 to 87)	96 (80 to 100)
⩾2500 cut point	92 (64 to 100)	87 (69 to 96)	75 (48 to 93)	96 (81 to 100)
⩾6000 cut point	85 (55 to 98)	100 (88 to 100)	100 (72 to 100)	94 (79 to 99)
Urine cytology positive‡	45 (17 to 77)	100 (88 to 100)	100 (48 to 100)	83 (66 to 93)

*Three cut points were used to demonstrate test performance under a range of circumstances: at the lower detection limit of the assay (fluorescence signal generated from 1500 proliferating HeLa S3 cells/well), where sensitivity of the test was maximal; at the point where the false-positive and false-negative rates of the test were equal (2500 cells/well); and at the point where the specificity of the test was 99% (6000 cells/well). CI = confidence interval; PPV = positive predictive value; NPV = negative predictive value.

†Excludes seven patients known to have prostate cancer on presentation.

‡Seven urine cytology samples were lost during processing and, consequently, were not included in analyses involving urine cytology (four patients with bladder cancer and three with nonmalignant disease).

Table 3. False-negative rates of the Mcm5 test and urine cytology by grade and stage of tumor and by urine volume*

	Tumor grade† (N)			Tumor stage† (N)			Urine volume, mL (N)		
Test	1 (17)	2 (23)	3 (21)	pTa (30)	pT1 (21)	pT2/pT3 (10)	<50 (14)	50–74 (29)	⩾75 (28)
Mcm5 test									
⩾1500 cut point, % (95% CI)	24 (7 to 50)	4 (0 to 22)	5 (0 to 24)	17 (6 to 35)	0 (0 to 16)	10 (0 to 45)	7 (0 to 34)	7 (1 to 23)	11 (2 to 28)
P		.10			.24			.78	
⩾2500 cut point, % (95% CI)	29 (10 to 56)	9 (1 to 28)	10 (1 to 30)	23 (10 to 42)	0 (0 to 16)	20 (3 to 56)	7 (0 to 34)	14 (4 to 32)	14 (4 to 33)
P		.12			.32			.64	
⩾6000 cut point, % (95% CI)	53 (28 to 77)	22 (7 to 44)	14 (3 to 36)	43 (25 to 63)	5 (0 to 24)	30 (7 to 62)	21 (5 to 51)	31 (15 to 51)	29 (13 to 49)
P		.011			.12			.73	
Urine cytology positive, % (95% CI)	88 (62 to 98)	48 (26 to 70)	35 (15 to 59)	76 (56 to 90)	35 (15 to 59)	25 (3 to 65)	—	—	—
P		.002			.002			—	

*Three cut points were used to demonstrate test performance under a range of circumstances: at the lower detection limit of the assay (fluorescence signal generated from 1500 proliferating HeLa S3 cells/well), where sensitivity of the test was maximal; at the point where the false-positive and false-negative rates of the test were equal (2500 cells/well); and at the point where the specificity of the test was 99% (6000 cells/well). Excludes seven patients known to have prostate cancer on presentation. All *P* values shown are obtained using the linear-by-linear association chi-squared test for trend with statistical significance set at the 5% level. CI = confidence interval; — = not applicable.

†Excludes tumors without tumor–node–metastasis grade or stage information *(25).*

Mcm5 value that is larger than that of a randomly selected patient without a malignancy.

Our analysis was performed using cut point values that corresponded to 1500 HeLa cells/well (lower detection limit of the assay), 2500 cells/well (equal false-positive and false-negative rates), and 6000 cells/well (specificity of 99% for all patients tested), which represented a wide range of performance levels (Table 2). At the 1500-cell cut point, the test had a 92% (95% CI = 83% to 97%) sensitivity and a 52% (95% CI = 43% to 61%) positive predictive value. At the 2500-cell cut point, the test had an 87% (95% CI = 77% to 94%) sensitivity and 64% (95% CI = 54% to 73%) positive predictive value. At the 6000-cell cut point, the test had a 72% (95% CI = 60% to 82%) sensitivity and a 96% (95% CI = 87% to 100%) positive predictive value. Importantly, where the specificity of the Mcm5 test was equal to that of cytology (97%), the immunofluorometric test was statistically significantly more sensitive than cytologic analysis of urine (73% [95% CI = 61% to 83%] versus 48% [95% CI = 36% to 60%], $P<.001$, McNemar's test; data not shown).

The number of tumors detected by urine cytology but not by the immunofluorometric Mcm5 test at the 1500-cell, 2500-cell, and 6000-cell cut points was one, two, and four tumors, respectively (data not shown). Conversely, the number of tumors missed by cytology but detected by the immunofluorometric Mcm5 test at the 1500-cell, 2500-cell, and 6000-cell cut points was 30, 28, and 20 tumors, respectively. The number of patients without a malignancy who were both correctly classified as not having a tumor by urine cytology and incorrectly classified as having a tumor by the immunofluorometric Mcm5 test at the 1500-cell, 2500-cell, and 6000-cell cut points were 60, 35, and two, respectively. Conversely, the number of patients without a malignancy who were correctly classified as not having a tumor by the immunofluorometric Mcm5 test at the 1500-cell, 2500-cell, and 6000-cell cut points but who were incorrectly classified as having a tumor by cytology were seven, seven, and seven, respectively.

On the basis of Multi-ROC analysis, the combination of the cytology and immunofluorometric Mcm5 tests offered limited

Table 4. False-positive rates of the Mcm5 test according to the presence of inflammation, benign prostatic hyperplasia, and calculi*

	Inflammation†		Benign prostatic hyperplasia‡		Calculi	
	No (n = 195)	Yes (n = 79)	Negative (n = 135)	Positive (n = 72)	Absent (n = 259)	Present (n = 16)
Mcm5 test						
⩾1500 cut point, % (95% CI)	22 (16 to 28)	23 (14 to 34)	21 (15 to 29)	21 (12 to 32)	21 (16 to 26)	44 (20 to 70)
⩾2500 cut point, % (95% CI)	12 (8 to 18)	14 (7 to 24)	13 (7 to 19)	13 (6 to 22)	12 (9 to 17)	19 (4 to 46)
⩾6000 cut point, % (95% CI)	1 (0 to 3)	1 (0 to 7)	0 (0 to 3)	1 (0 to 8)	0 (0 to 2)	6 (0 to 30)

*Three cut points were used to demonstrate test performance under a range of circumstances: at the lower detection limit of the assay (fluorescence signal generated from 1500 proliferating HeLa S3 cells/well), where sensitivity of the test was maximal; at the point where the false-positive and false-negative rates of the test were equal (2500 cells/well); and at the point where the specificity of the test was 99% (6000 cells/well). Excludes seven patients known to have prostate cancer on presentation. CI = confidence interval.

†Inflammation includes urinary tract infection and acute chronic cystitis.

‡Males only.

Table 5. Immunofluorometric Mcm5 test performance in patient groups after further follow-up*

Patient groups and subgroups	Group size (N)	Median signal	Interquartile range	Percentage of signals ⩾1500 (95% CI)
Negative for cancer	266	<1500	1500–<1500	20 (15 to 25)
Calculi	16	<1500	<1500–2388	44 (20 to 70)
No calculi	250	<1500	<1500–<1500	18 (13 to 23)
BPH (males)	70	<1500	<1500–<1500	19 (10 to 30)
No BPH (males)	131	<1500	<1500–<1500	18 (12 to 26)
Prostate cancer	12	2720	1902–3794	92 (62 to 100)
Bladder cancer	71	8927	4064–27 874	92 (83 to 97)
G1	18	3944	2012–6948	78 (52 to 94)
G2/G3	42	16 847	6324–37 480	95 (84 to 99)
CIS	7	8927	4713–40 148	100 (59 to 100)
Nontransitional	4	30 525	9461–122 041	100 (40 to 100)

*Four renal transitional cell carcinomas are not included in the table: two were G2pT1 and had Mcm5 test signals of 33,181 and 28,093; one was G3pT1 and had a Mcm5 test signal of 11,423; one was G2pTa and had an Mcm5 test signal of 4,744. CI = confidence interval; BPH = benign prostatic hyperplasia; CIS = carcinoma *in situ.*

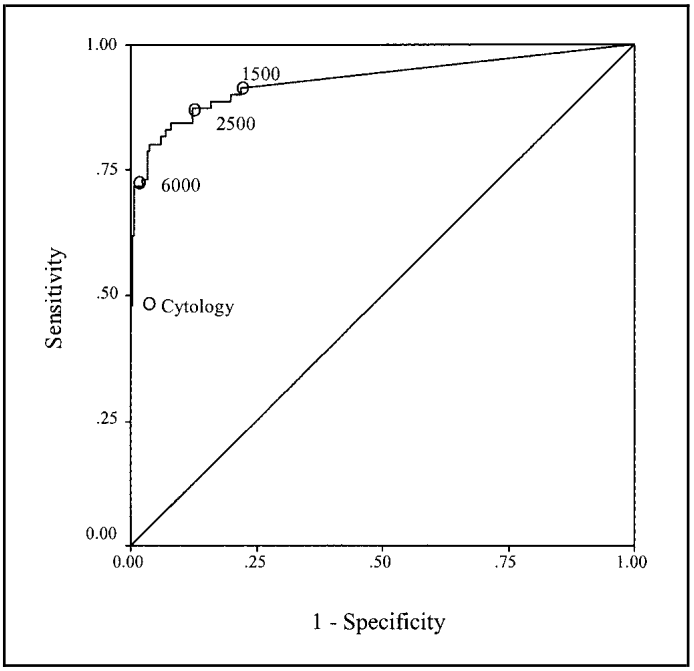

Fig. 1. Receiver operating characteristic curve of immunofluorometric minichromosome maintenance 5 test. The **jagged curve** is the nonparametric receiver operating characteristics curve. The **diagonal line** is a reference line. Points are marked to demonstrate the sensitivity and 1-specificity of urine cytology (Cytology) and of the Mcm5 test at cut points of 1500, 2500, and 6000 cells/well (area under curve = 0.93 [95% confidence interval = 0.89 to 0.97]).

increases in performance relative to the immunofluorometric test in isolation (at the 6000-cell cut point, the sensitivity of the test could be improved from 72% [95% CI = 60% to 82%] to 78% [95% CI = 66% to 87%] at the expense of a reduction in specificity, from 99% [95% CI = 97% to 100%] to 97% [95% CI = 94% to 98%]; data not shown). Conversely, in those patients indicated to be without tumor by cytology, the immunofluorometric Mcm5 test discriminated effectively between patients with and without bladder cancer, as demonstrated by the large area under the Multi-ROC curve (0.89 [95% CI = 0.79 to 0.94]), which is statistically significantly larger than the area assumed by the null hypothesis (0.5) ($P<.001$). The immunofluorometric test detected primary bladder cancer and recurrent bladder cancers equally well at all three cut points; the estimates of the areas under the ROC curves for primary bladder cancer and recurrent bladder cancer were 0.91 and 0.96, respectively ($t = 1.08$, $P = .28$).

We compared the false-negative rates of the immunofluorometric Mcm5 test and urine cytology according to tumor grade and stage (Table 3). There was evidence of a decreasing trend in the false-negative rate with increasing tumor grade and stage for both cytologic analysis of urine and the immunofluorometric Mcm5 test. For example, although the sensitivity of cytology was 48% (95% CI = 35% to 60%) for all tumor grades combined (Table 2), the false-negative rate was 88% (95% CI = 62% to 98%) for G1 transitional cell carcinomas and 35% (95% CI = 15% to 59%) for G3 transitional cell carcinomas. For G1 transitional cell carcinomas, the false-negative rate of cytology (88%) was statistically significantly higher than that of the Mcm5 test at the 1500-cell cut point (false-negative rate = 24%, 95% CI = 7% to 50%; $P = .006$) but was not statistically significantly higher than that of the Mcm5 test at the 6000-cell cut point (false-negative rate = 53%, 95% CI = 28% to 77%; $P = .07$) (Table 3).

Interestingly, the amplitude of the Mcm5 immunofluorometric signal was associated with the grade of the transitional cell carcinoma. For example, the median immunofluorometric signal for G2 and G3 transitional cell carcinomas combined was 16 847 (interquartile range = 6324 to 37 480), whereas the median

signal for G1 transitional cell carcinomas was 3944 (interquartile range = 2012 to 6948) ($P<.001$, Mann–Whitney U test). We found no evidence for an association between the false-negative rate of the immunofluorometric Mcm5 test at any cut point and the volume of the voided urine specimen (Table 3). For example, when we categorized urine volumes into increasing thirds (representing 30%, 37%, and 33% of transitional cell carcinoma case patients, respectively), the false-negative rates of the immunofluorometric Mcm5 test at the 1500-cell cut point were 7%, 7%, and 11% for urine sample volumes less than 50 mL, 50–74 mL, and 75 mL or more, respectively ($P = .78$). The false-negative rates of the immunofluorometric Mcm5 test at the 6000-cell cut point were 21%, 31%, and 29% for urine sample volumes less than 50 mL, 50–74 mL, and 75 mL or more, respectively ($P = .73$).

A major constraint on the use of proliferation markers for the diagnosis of cancer is their potential for producing false-positive results in hyperplastic or reactive inflammatory conditions *(6,34).* We found no evidence at any cell cut point for an association between the false-positive rate of Mcm5 immunofluorometry and the presence of either urinary tract inflammation or benign prostatic hyperplasia in the tested individual (Fisher's exact test; Table 4). For example, at the lower detection limit of the test (i.e., the 1500-cell cut point), the false-positive rates were not statistically significantly different between those with (23%, 95% CI = 14% to 34%) and those without (22%, 95% CI = 16% to 28%) inflammation ($P = .87$) or between those with (21%, 95% CI = 12% to 32%) and those without (21%, 95% CI = 15% to 29%) benign prostatic hyperplasia ($P = 1$). At the lower detection limit of the test (i.e., the 1500-cell cut point), the false-positive rate was somewhat higher for patients who were diagnosed with calculi (44%, 95% CI = 20% to 70%) than it was for those without calculi (21%, 95% CI = 16% to 26%), but the difference was not statistically significant ($P = .056$) (Table 4).

The diagnostic utility of the immunofluorometric Mcm5 test in the primary diagnosis of genito-urinary cancer was highlighted by the results of subsequent clinical follow-up exams. Those additional clinical investigations revealed that 11 of the 61 patients whose immunofluorometric Mcm5 test results were initially categorized as false positives did in fact have clinically significant disease. Of those patients, five had prostate cancer, three had bladder cancer (two had a G1pTa transitional cell carcinoma and one a G2pT1 transitional cell carcinoma), and one had a transitional cell carcinoma of the renal pelvis (G2pTa). Two patients had urinary tract calculi: One had a renal calculus and the other had a bladder calculus.

Table 5 shows the performance of the immunofluorometric Mcm5 test according to the diagnosis made at the clinical follow-up. Male patients who were diagnosed with prostate cancer were more likely ($P<.001$) to have Mcm5 levels above the 1500-cell cut point (92%, 95% CI = 62% to 100%) than patients without malignancy (20%, 95% CI = 15% to 25%) (Table 5). However, we could not accurately assess the sensitivity and specificity of the immunofluorometric Mcm5 test for the diagnosis of prostate cancer because prostate biopsies were not available for all of the male patients in our study. Among the 12 patients diagnosed with prostate cancer at presentation or identified during the follow-up (11 of whom were identified by the Mcm5 test), one had a total serum PSA level less than 4 ng/mL, three had PSA levels ranging from 4 to 10 ng/mL, four had PSA levels ranging from 10 to 20 ng/mL, and four had PSA levels ranging from 32.5 to 50 ng/mL. All of the patients with prostate cancer had organ-confined disease, except for one who had pelvic lymph node metastases. That patient had a total serum PSA level of 50 ng/mL. Interestingly, the median Mcm5 immunofluorometric signal for patients with prostate cancer was 2720, which was similar to the median signal for patients who were diagnosed with G1 transitional cell carcinomas (3944) (Table 5). The Mcm5 signal was statistically significantly higher for patients with prostate cancer (median signal = 2720) than for patients with benign prostatic hyperplasia (median signal <1500; $P<.001$, difference in median signals >1220, 95% CI>473 to 1512). The immunofluorometric Mcm5 signal was statistically significantly higher ($P<.001$) for patients with either G2 or G3 transitional cell carcinomas (median signal = 16 847) than for those with G1 transitional cell carcinomas (median signal = 3944; $P<.001$, difference in median signals = 12 903, 95% CI = 4245 to 19 774). The percentage of Mcm5 signals greater than or equal to that at the 1500-cell cut point was statistically significantly higher for patients with calculi (44%) than for those without calculi or malignancies (18%; $P = .014$, difference in percentages = 26%, 95% CI = 2% to 51%). The highest immunofluorometric Mcm5 values were seen in patients with urinary tract malignancies of nontransitional origin (median = 30 525) (Table 5). In addition to bladder and prostate cancer, the immunofluorometric Mcm5 test was able to detect renal transitional cell carcinoma (Table 5).

DISCUSSION

Many deaths from bladder cancer could be prevented by early detection of new tumors and vigilant surveillance for recurrences *(35).* Therefore, there is an urgent need for a diagnostic test that is less invasive than cystoscopy but more sensitive than cytology. Here we have shown that immunofluorometric quantification of the DNA replication initiation factor Mcm5 in urine sediments provides a simple, noninvasive method to diagnose primary and recurrent bladder cancers with high sensitivity and specificity (Table 5). Interestingly, high-grade urothelial neoplasms, including G2/G3 transitional cell carcinomas and carcinoma *in situ,* which are associated with poor prognoses, were associated with statistically significantly higher immunofluorometric Mcm5 signals than low-grade urothelial neoplasms, such as G1 transitional cell carcinomas (Table 5), indicating that the amplitude of the Mcm5 immunofluorometric signal on presentation may be of prognostic value.

Our data show clearly that the sensitivity of the immunofluorometric Mcm5 test for detecting bladder cancers, particularly low-grade transitional cell carcinomas, was superior to that of urine cytology. Thus, use of the immunofluorometric Mcm5 test could circumvent the exhaustive expert interpretation that is typically required for conventional cytologic analysis of urine samples. Although other markers, such as nuclear matrix protein 22, bladder tumor antigen, and telomerase, are also more sensitive for the diagnosis of bladder cancer than urine cytology, they are compromised by their low specificities. The high false-positive rates for those markers have been linked to inflammation, benign prostatic hyperplasia, and other nonmalignant urologic conditions *(6,34).* Importantly, inflammatory conditions (e.g., urinary tract infection, acute and chronic cystitis, and bacillus Calmette-Guèrin-induced granulomatous cystitis) and benign prostatic hyperplasia were not associated with false-

positive results in the immunofluorometric Mcm5 test (Table 4). In some cases, bladder and renal calculi were associated with false-positive Mcm5 immunofluorometric results, possibly because trauma to the urothelial lining exposes the stem-transit compartment to urine, which, when coupled with reparative growth, may result in shedding of reactive Mcm5-positive cells into urine *(23)*.

The immunofluorometric Mcm5 test, at the levels of sensitivity and specificity observed in this study (Table 2), could facilitate the initial diagnosis and follow-up of patients with transitional cell carcinoma. It is important to note that the cut points of the assay can be altered to allow the test to be used in different patient populations. For example, frequent cystoscopy is currently used to monitor for recurrent superficial disease. However, that procedure is complicated by the fact that it can cause urinary tract infections and urethral damage, and many patients find it uncomfortable. The Mcm5 test, applying a 1500-cell cut point (92% sensitivity, versus 48% for cytology) would be useful in identifying patients with superficial low-grade disease and in planning and reducing the frequency of subsequent cystoscopies in these patients. At this cut point, the Mcm5 test (negative predictive value 97%), would be beneficial for monitoring therapeutic response in these patients while they are undergoing chemotherapy and immunotherapy. Applying the 6000-cell cut point (99% specificity, 96% positive predictive value) would be more applicable than lower cut points for screening populations at high risk for bladder cancer and would also provide improved performance (e.g., 86% sensitivity for G3 transitional cell carcinomas) over conventional cytology (e.g., 65% sensitivity for G3 transitional cell carcinomas) for the detection of high-grade disease (Table 3) where specificity is equivalent. Improvements in the performance of the Mcm5 assay to reduce the false-negative rate and extensive comparative clinical studies with sufficient follow-up are required to determine whether the immunofluorometric Mcm5 test can replace, or at least reduce the frequency of, cystoscopy in the management of patients with hematuria who are at high risk for transitional cell carcinomas.

This diagnostic test for urothelial neoplasia targets the evolutionarily conserved step of DNA replication initiation, which lies at the convergence point of complex and branched oncogenic signaling pathways. This critical step in growth regulation should be common to all tumor types; therefore, detecting the MCM constituents of the DNA replication initiation pathway may aid in the diagnosis of other tumor types *(17)*. Indeed, we have previously reported that using MCM-specific antibodies to label dysplastic and malignant cells in cervical smears can improve the sensitivity of the Pap test for cervical cancer *(21)*. Our finding that four of the bladder tumors detected in this study were of nonurothelial origin (i.e., a non-Hodgkin's B cell lymphoma, an extramedullary myelomonocytic leukemia, an invasive sigmoid adenocarcinoma, and a primary small cell neuroendocrine carcinoma) reinforces the potential utility of this diagnostic strategy for detecting cancers of different origins.

We intended to apply the Mcm5 test to the diagnosis of bladder cancer; however, our observation that the test appeared to detect prostate cancer in addition to bladder cancer highlights the potential generality of this novel diagnostic strategy. Although prostate cancer is the second most common cause of cancer mortality for men in industrialized countries, its cause is unclear, and efforts to decrease the morbidity and mortality associated with this disease are focusing on secondary prevention through screening and early treatment *(36)*. Proponents of prostate cancer screening assert that early detection can lead to the discovery of organ-confined disease, which has a greater potential for cure than disease that is not confined to the prostate *(37)*. Indeed, declining mortality rates among men diagnosed with prostate cancer in the United States have been attributed to the introduction of serum PSA testing. However, the effectiveness of the PSA test in prostate cancer screening remains controversial because age-specific mortality rates have similarly declined in Western countries that do not have active PSA screening programs *(38)*. A major constraint of the PSA test is that PSA levels can be elevated in benign conditions; for example, up to 25% of individuals with benign prostatic hyperplasia have elevated PSA levels *(39)*. Consequently, at a cut-off of 4 ng/mL, PSA testing has a sensitivity of only 35% and a specificity of 63% *(40)*.

In this context, two important observations from our study were that patients with prostate cancer had elevated levels of Mcm5 protein in their urine sediments compared with males without bladder or prostate malignancy and that elevated levels of Mcm5 were not associated with benign prostatic hyperplasia. These findings should be viewed as preliminary because they are based on only 12 patients. However, they are consistent with findings from our recent immunohistochemical studies of MCM protein expression patterns in benign and malignant prostate tissue. In normal prostate and benign prostatic hyperplasia, MCM protein expression is restricted to 2% of the basal glandular epithelial cells. By contrast, in prostatic adenocarcinoma, 20%–30% of cells constituting the malignant glandular epithelium express MCM proteins *(41)*. This malignant glandular epithelium is composed of a monolayer of cells, such that the MCM-expressing cells are present in an ectopic luminal location that facilitates their exfoliation into luminal secretions. Further large-scale clinical studies that compare levels of Mcm5, serum PSA, and the ratio of free to total PSA in men with early-stage histologically confirmed, localized prostate cancer with those in men without malignancy will be required to evaluate the potential utility of the immunofluorometric Mcm5 test in prostate cancer screening and diagnosis.

In conclusion, we have demonstrated that immunofluorometric detection of Mcm5 in urine sediments is a sensitive and specific diagnostic test for bladder cancer. The test detects bladder cancers of all stages and grades, including those often missed by urine cytology. It also may detect early-stage, organ-confined prostate cancer. This simple and noninvasive method for detecting genito-urinary tract malignancy is readily automated and has many potential clinical applications, including primary diagnosis, monitoring for relapse, measurement of therapeutic response, and population screening. Finally, the detection of different tumor types highlights the generality of this diagnostic approach and suggests potential applicability of the test to other epithelial-lined organ systems.

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