Quantitative measurement of telomerase reverse transcriptase mRNA and chromosomal analysis of urine by M-FISH in the diagnosis and follow-up of bladder cancer

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Abstract. This study was performed to evaluate the clinical utility of the measurement of the expression of telomerase enzyme (the catalytic subunit of the complex hTERT) and of the chromosomal analysis of urine by multi-color fluorescence *in situ* hybridization (M-FISH) assay for the detection of bladder cancer and its recurrence. These results were compared with those afforded by urine cytology, hematuria screening and the bladder tumor antigen (BTA) and fibrin degradation products (FDP) tests. Urine samples were obtained from three groups: 30 patients with bladder cancer, 15 patients with non-malignant bladder disorders and 8 healthy individuals. hTERT mRNA was measured by reverse transcription real-time PCR. M-FISH was performed using a mixture of fluorescent labeled probes for the centromeric regions of chromosomes 3, 7, 17, and the locus specific identifier p16 probe for the 9p21 locus. We demonstrated that the overall sensitivity of hemoglobin dipstick and the BTA and FDP tests was insignificantly greater than that of urine cytology, but with a lower specificity. The hTERT mRNA expression marker offered significantly greater sensitivity for detecting all bladder cancers, especially superficial and low grade tumors, than did urine cytology, and with a higher specificity. M-FISH was significantly more sensitive than urine cytology in detecting bladder tumors, but had lower specificity (insignificant results). The superior sensi-

tivity of M-FISH was maintained when comparing the two assays in terms of low-stage and low grade tumor detection. M-FISH was the most sensitive and specific test in detecting tumor recurrence, followed by the BTA test. hTERT, hematuria screening and FDP showed relatively low sensitivity and low specificity during follow-up. Our findings suggest that the assessment of hTERT expression and chromosomal abnormalities in urine represent reliable tools - equally specific yet far more sensitive than conventional cytology - for the early detection of bladder cancer. The high sensitivity of FISH in detecting recurrence makes it useful for reducing the number of cyctoscopies usually performed in the accurate follow-up of these cases.

Introduction

Bladder cancer is one of the most prominent cancers in the world, and one of the most prevalent cancers in Egypt. The National Cancer Institute (NCI), Cairo, reported that bladder cancer constitutes 30.3% of all cancers in Egypt (1,2). As Egypt represents a hyperendemic area for schistosome infection, schistosome-related bladder cancer accounts for 82.4% of all bladder cancers (3,4). Almost 30-50% of newly-diagnosed patients have superficial tumors limited to the mucosa or submucosa. Such cases, when the disease is detected before it invades the bladder muscle, usually have a good prognosis. Unfortunately, the recurrence rate of bladder cancer is 50-70%, and 20-30% of recurrent tumors progress to muscle invasive disease. Consequently, patients must receive regular follow-up exams at 3-6 month intervals (5).

The current methods of evaluation for initial diagnosis and follow-up involve cystoscopy and biopsy, an invasive procedure. It may also be inconclusive at times, especially in the numerous patients with an indwelling catheter or active inflammation, where it may not be definitive due to the grossly abnormal appearance of the bladder mucosa. Cytoscopy is therefore not ideal for the screening and follow-up of bladder cancer; what is needed is a non-invasive method with high sensitivity, specificity and predictive values for bladder cancer detection, and for the identification of patients at high risk of tumor recurrence or progression (6,7).

There are several situations during diagnosis and the selection of appropriate treatment for bladder cancer where

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objective markers could potentially transform clinical practice. Several attempts have been made to improve the detection of cancer cells in urine samples by multiple urinary tumor markers, such as nuclear matrix protein (NMP)22, DNA ploidy and cytokeratins; however, the sensitivity of these markers is too low to be introduced to clinical practice, indicating the need for new markers (8). Molecular markers are now a source of great hope. Identification of the genetic and chromosomal changes that are fundamental to the malignant process in bladder cancer would be useful for developing detection and preventive strategies, while identifying those changes that are secondary to the malignant process may be more valuable for prognostic purposes. Awareness of both is likely needed in order to develop effective therapeutic approaches (9).

Bladder cancer initiation and development involves genetic changes and chromosomal aberrations that often lead to either the deletion of tumor suppressor genes or the induction of oncogenes. This increases the proliferative potential of tumor cells and their resistance to apoptosis. However, these processes alone do not ensure tumor growth. After successive replication, cells lose their protective telomere ends, resulting in chromosomal shortening and cell death. Malignant cells are able to maintain telomere length above a critical threshold by up-regulating the expression of the enzyme telomerase, which catalyzes the addition of telomeric repeats to the 3'-end of the chromosome to allow unlimited replication potential (9). Telomerase, a ribonucleoprotein enzyme, needs at least two components to be functional: the RNA component, which is coded by the human telomerase RNA (*hTR*) gene and serves as a template for telomere DNA synthesis, and the human telomerase reverse transcriptase subunit (*hTERT* gene), which codes for the catalytic subunit of the enzyme. Telomerase activity is the most general molecular marker for the identification of human cancer and can be detected in 85% of all tumors, whereas little or no telomerase expression is found in healthy tissues. A method for the quantitative measurement of *hTERT* mRNA expression may be of import to molecular diagnosis in tumors and corresponding bodily samples (10).

Bladder tumor initiation and progression is accompanied by increased chromosomal instability and aneuploidy. Cytogenetic studies on bladder cancer have revealed frequent alterations of a variety of chromosomes, including chromosomes 9, 17, 7, 11, 1, 8 and others. Fluorescence *in situ* hybridization (FISH) can be used to detect cells with these chromosomal alterations (11).

In the present study, we quantified telomerase reverse transcriptase mRNA and screened for the presence of chromosomal aberration involving chromosomes 3, 7, 17, and 9 in the urine of bladder cancer patients and those at risk of developing bladder cancer. This was in order to shed light on their clinicopathological significance in the diagnosis and follow-up of the disease, and examine their relation to other routine diagnostic markers that use a non-invasive route.

Patients and methods

Subject selection. The study was conducted on 45 patients, with 8 apparently healthy individuals serving as the control group. All patients were selected from the Urology department of Tanta University, and consent was obtained from all subjects included in the study. The 45 patients included 15 with non-neoplastic bladder disorders (6 of whom were diagnosed with

Bilharzial cystitis, 3 with non-Bilharzial chronic cystitis and 6 with bladder stones and cystitis). Of these, 11 were male and 4 female, with ages ranging 40-58 years (mean 52.8±5.1). The other 30 patients were newly diagnosed with bladder cancer and included 21 males and 9 females, with ages ranging 46-71 years (mean 56.7±7.5). Of these, 9 were diagnosed with Bilharzial squamous cell carcinoma (SCC), 7 with Bilharzial transitional cell carcinoma (TCC) and 14 with non-Bilharzial TCC. Patients were divided according to disease stage: 13 cases of superficial carcinoma (3 pTa and 10 pT1) and 17 of invasive tumors (pT2-pT4). In terms of histopathological grade, 6 patients were grade I, 13 were grade II and 11 were grade III. None of the patients had a history of upper urinary tract disorders, as confirmed by radiological and laboratory examinations. The diagnosis of bladder cancer was based on clinical, radiological, cystoscopy and histological examinations. Those cases associated with cyctitis were first subjected to urine culture and sensitivity tests for proper antibiotic treatment before the initiation of the study. The control group included 6 males and 2 females, with ages ranging 40-63 years (mean 51±8.2).

All subjects involved in the study were subjected to routine examinations, including clinical examination, radiological studies, urine analysis, kidney function tests and the indirect heamagglutination test for Schistosomiasis. They also underwent specific examinations, including cyctoscopy and biopsy for urinary bladder masses, urine cytological examination, hematuria screening by hemoglobin dipsticks (Hemastix, Bayer Diagnostic, USA), detection of bladder tumor antigen in urine using the bladder tumor antigen (BTA) stat test (Polymedco Inc., USA), detection of fibrin degradation products (FDP) in urine using the latex semi-quantitative thrombo-Wellcotest kit (Murex, USA), a quantitative assay of telomerase reverse transcriptase mRNA (hTERT mRNA) in urine using real-time RT-PCR and screening for aberrations, including chromosomes 3, 7, 17 and the 9p21 locus in urine samples, by UroVysion multi-color fluorescence *in situ* hybridization (M-FISH).

Specimen collection. Morning urine samples were collected for cytological examination. About 100 ml of the second morning samples were collected into autoclaved collection tubes for routine and specific investigations. Each urine sample was divided as follows: 10 ml for routine urine analysis, 5 ml for culture and sensitivity if indicated, 2 ml for hemoglobin dipstick, 2 ml for FDP detection, a few drops for the BTA stat test, 25 ml for RNA extraction and subsequent hTERT mRNA quantitation, and 50 ml for FISH.

Patient follow-up. Follow-up was done at one month and then every three months for one year following management. For the first two visits, follow-up consisted of cystoscopy, urine cytology, hemoglobin dipstick, the FDP test, the BTA stat test, quantitative assay of hTERT mRNA, and chromosomal assay. Subsequently, follow-up was done by cystoscopy every three months when possible.

Quantitation of hTERT mRNA. Quantitation of hTERT gene expression was performed by two-step quantitative real-time RT-PCR. In the reverse transcription step, cDNA was reverse transcribed from extracted RNA using random hexamers. In

the real-time polymerase chain reaction step, products were amplified from cDNA using the AmpliTaq Gold DNA polymerase, TaqMan PDARs primers and TaqMan fluorescent probe (Applied Biosystems, USA).

Total-RNA extraction. Total-RNA was extracted from cell pellets harvested from the urine samples using a combination of TRIzol reagent and the Qiagen RNeasy mini spin column (RNeasy Mini Kit, Qiagen, USA) as described previously. Briefly, TRIzol and chloroform were added to the cell pellet, which was then incubated for 15 min and centrifuged. The upper aqueous phase was transferred to the RNeasy mini spin column. The columns were washed and RNA was eluted in RNase-free water (12,13).

Real-time quantitative RT-PCR. For the quantitation of hTERT mRNA using real-time RT-PCR, a calibration curve of human reference total-RNA (Applied Biosystems) was used. Reference total-RNA is composed of total-RNA from 10 human cell lines, with quantities of RNA from each cell line optimized for maximum representation of hTERT mRNA. In order to normalize sample-to-sample differences in RNA input, RNA quality and RT efficiency, the level of glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) expression was quantitatively measured in each sample with a GAPDH standard curve.

First-step PCR (cDNA synthesis). cDNA was reverse transcripted from the total-RNA of the urine samples and from the stock RNA of the human reference total-RNA using random hexamers (High-Capacity cDNA kit, Applied Biosystems) under the following conditions: hexanucleotides annealing for 10 min at 25˚C, cDNA synthesis for 30 min at 48˚C, then enzyme inactivation for 5 min at 95˚C. The cDNA of the subjects was stored at -70˚C until use. Two sets of 2-fold dilution series (50-3.125 ng/μl) of human reference cDNA were prepared in RNase-free H$_2$O and stored at -70˚C for later use (14).

Second-step PCR (cDNA amplification). AmpliTaq Gold DNA polymerase was used to amplify cDNA using TaqMan primers, TaqMan fluorescent probes and TaqMan universal PCR master mix (Applied Biosystems). The sequences of the primers and of the hTERT probe were: forward primer, 5'-CGGAAGAGTGTCTGGAGCAA-3'; reverse primer, 5'-GGATGAAGCGGAGTCTGGA-3'; probe: Fam-TGCAAAGCATTGGAATCAGACAGCACT-Quencher MGB. Amplification of GAPDH was performed for each sample in a separate tube using the following primers and probe: forward primer, 5'-GAAGGTGAAGGTCGGAGTC-3'; reverse primer, 5'-GAAGATGGTGATGGGATTTC-3'; probe: VIC-CAAGCTTCCCGTTCTCAGCC-TAMRA.

The TaqMan universal PCR master mix contains AmpliTaq Gold DNA polymerase, AmpErase UNG, dNTPs with dUTP, and optimized buffer. The reaction mixture was performed according to the manufacturer's instructions in a final volume of 50 μl under the following conditions: initial UNG incubation at 50˚C for 2 min, enzyme activation at 95˚C for 10 min, 40 cycles of denaturation at 95˚C for 15 sec, and annealing/extension at 60˚C for 1 min. The assay was performed utilizing the GeneAmp 5700 Sequence Detection System (Applied Biosystems). The amounts of hTERT and GAPDH mRNA were determined from the corresponding standard curves and the hTERT mRNA was divided by the corresponding GAPDH reference amount to obtain a normalized hTERT mRNA value (15).

Detection of aberration in chromosomes 3, 7, 17 and the 9p21 locus. M-FISH (UroVysion Kit, Vysis, USA) was designed to detect aneuploidy in chromosomes 3, 7 and 17, and the loss of the 9p21 locus in urine. The mixture consists of four labeled probes. These include three α-satellite repeat sequence probes: chromosome enumeration probe (CEP)3 spectrum red, CEP7 spectrum green and CEP17 spectrum aqua, which hybridize to the centromer regions of chromosomes 3, 7 and 17 respectively, and a unique sequence probe: locus specific identifier p16 spectrum gold, which hybridizes to the p16 gene at the 9p21 locus. Cell pellets from fresh urine samples were fixed on slides. DNA was denatured to its single-strand form and allowed to hybridize with the UroVysion probes. Following hybridization, the unbound probes were removed by a series of washes and the nuclei were counterstained with 4',6-diamidino-2-phenylindol (DAPI) fluorescent stain. The probes were then viewed on an Olympus fluorescence microscope BX60, equipped with a filter set including Aqua single bandpass (chromosome 17), Gold single bandpass (the 9p21 locus) and Red/Green/DAPI triple bandpass (chromosomes 3 and 7) (11,16).

The slides were scored for hybridization signals according to the manufacturer's protocol. Enumeration of FISH signals was done on target cells that appeared morphologically abnormal (large nuclear size, irregular nuclear shape and patchy DAPI staining). Cells were defined as FISH-positive if they showed three or more signals of two or more of the Aqua (CEP 17), Green (CEP 7) or Red (CEP 3) probes and/or a loss of both gold signals (9p21). For each specimen, a minimum of 25 cells was analyzed. If fewer than 25 target cells were found, the remaining cells were marked as disomic (i.e., 2-2-2-2). Control slides were run concurrently with patient slides to monitor assay performance and to assess the accuracy of signal viewing (UroVysion control slides, Vysis).

The cut-off for FISH-positive bladder cancer specimens was performed according to the criteria suggested by Mayo's Cytogenetics Laboratory, 2002. Accordingly, a specimen was considered FISH-positive for bladder cancer if at least one of the following criteria was met: i) ≥5 of the 25 examined cells exhibiting a gain of 2 or more chromosomes (3, 7 or 17) in the same cell, or ii) ≥12 of the 25 cells exhibiting zero 9p21 signals (both copies lost) (17).

Results

Utility of urine cytology, hematuria, BTA and FDP screening in the detection of bladder cancer. Forty-five patients (15 with benign bladder disorders and 30 with bladder cancer) and 8 healthy individuals were included in the study. Their urine samples were subjected to cytological examination, the measurement of BTA, hematuria and FDP (Tables I and II).

Cytological examination was negative for malignancy in all control subjects and in 13 (86.6%) of the 15 benign cases.

Table I. Significance of urine markers in the diagnosis of bladder cancer.

	Cytology	Hb Dipstick	BTA stat	FDP	hTERT mRNA	M-FISH
Sensitivity (%)	53.3	76.7	70.0	70.0	93.3	90.0
Specificity (%)	92.5	14.3	64.3	71.4	100.0	85.7
PPV (%)	94.1	65.7	80.7	84.0	100.0	93.1
NPV (%)	48.1	22.2	50.0	52.6	87.5	80.0
Accuracy (%)	85.3	56.8	68.2	70.4	95.5	88.6

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

Table II. Sensitivity of the different urine markers studied to various histopathological features of bladder cancer.

Histopathological features	Cytology (%)	Hb Dipstick (%)	BTA stat (%)	FDP (%)	hTERT (%)	M-FISH (%)
Pathological stage						
Superficial tumors						
pTa (n=3)	0.0	33.3	33.3	33.3	66.7	33.3
pT1 (n=10)	50.0	60.0	50.0	60.0	90.0	90.0
Invasive tumors						
(pT2-4) (n=17)	64.7	94.1	88.2	82.3	100.0	100.0
Pathological grade						
I (n=6)	16.7	33.3	16.7	33.3	83.3	50.0
II (n=13)	53.8	84.6	69.2	69.2	92.3	100.0
III (n=11)	72.7	90.9	100.0	90.9	100.0	100.0
Pathological type						
Bilharzial SCC (n=9)	44.4	77.8	55.6	77.8	100.0	100.0
Bilharzial TCC (n=7)	57.1	85.7	71.4	71.4	85.7	85.7
Non-Bilharzial TCC (n=14)	57.1	85.7	78.6	64.3	92.9	85.7

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

Two benign cases were suspected of having malignant cells by urine cytology. This was disproved by biopsy and histopathological examination. Urine cytology was positive for malignancy in 16 (sensitivity 53.3%) of the 30 patients with bladder cancer examined. It afforded an accuracy of 85.3%, a specificity of 92.5%, a positive predictive value (PPV) of 94.1% and a negative predictive value (NPV) of 48.1%. Urine cytology was markedly insensitive in detecting superficial tumors (a sensitivity of 0 and 50% for tumor stages pTa and pT1, respectively). Its sensitivity increased to 64.7% when detecting invasive tumors (P>0.05). Similarly, the sensitivity of urine cytology was very low when detecting grade I tumors (16.7%), but increased to 53.8 and 72.7% for tumor grades II and III, respectively (P>0.05). No significant difference was observed in the sensitivity of urine cytology to the different pathological tumor types (44.4% for Bilharzial SCC and 57.1% for both Bilharzial and non-Bilharzial TCC, P>0.05).

Hemoglobin was measured in urine by semi-quantitative Hemastix assay. None of the control subjects were positive for urine hemoglobin. The test was positive in 12/14 (85.7%) of the patients with benign disorders and in 23/30 (76.6%) of the bladder cancer cases. Despite the considerable sensitivity of the test in detecting bladder cancer (76.6%), it showed very poor specificity (14.3%), accuracy (56.8%), PPV (65.7%) and NPV (22.2%). As was the case with urine cytology, urine hemoglobin was significantly insensitive in detecting superficial tumors (sensitivity 33.3, 60 and 94.1% for pTa, pT1 and invasive tumors, respectively; P<0.05). Similarly, the test was significantly more sensitive to higher tumor grades than lower ones [sensitivity 33.3 (P<0.05), 84.6 and 90.9% (P>0.05) for tumor grades I, II and III, respectively]. No difference was observed in the sensitivity of the hemoglobin test to the different histological tumor types (sensitivity 77.8% for Bilharzial SCC and 85.7% for Bilharzial and non-Bilharzial TCC; P>0.05).

All control subjects were negative for urine BTA. The antigen was detected in the urine of 5 (35.7%) benign cases and 21 (70%) bladder cancer cases. The specificity of the test for

the diagnosis of bladder cancer was 64.3%, the PPV 80.7%, the NPV 50% and the accuracy 68.2%. The test had a significantly higher sensitivity in detecting invasive tumors compared to superficial ones, and in detecting high grade tumors compared to low grade ones [sensitivity 33.3, 50 and 88.2% for tumor stages pTa, pT1 and pT2-4, respectively ($P<0.05$), and 16.7, 69.2 and 100% for tumor grades I, II and III, respectively ($P<0.05$)]. No significant difference in BTA sensitivity to different histological types (55.6, 71.4 and 78.6% for Bilharzial SCC, Bilharzial TCC and non-Bilharzial TCC, respectively; $P>0.05$) was observed.

Similar to the previous assays, urine FDP was positive in 4 (26.5%) benign cases and 21 (70%) bladder cancer cases. It had a specificity of 71.4%, a PPV of 84%, an NPV of 52.6% and an accuracy of 70.4% in detecting bladder cancer. It showed lower sensitivity when detecting low grade and superficial tumors, but no difference when detecting different pathological types [sensitivity 33.3, 60 and 82.3% for pTa, pT1 and pT2-4, respectively ($P>0.05$), 33.3, 69.2 and 90.9% for grades I, II and III, respectively ($P>0.05$), and 77.8, 71.4 and 64.3% for Bilharzial SCC, Bilharzial TCC and non-Bilharzial TCC, respectively ($P>0.05$)].

Expression of hTERT mRNA in bladder cancer. hTERT mRNA was measured in urine sedimented cells using quantitative real-time PCR and normalized to GAPDH expression. None of the healthy individuals expressed hTERT. Four (26.6%) of the 15 benign cases showed very low hTERT expression (range 0.03-0.57; mean 0.12±0.1) with no significant difference compared to the controls ($P>0.05$). Three of the 4 benign cases expressing hTERT showed cytologic atypia. On the other hand, 28 (93.3%) of the 30 bladder cancer cases demonstrated significantly high hTERT expression (range 0.33-13.58; mean 4.3±3.01; $P<0.05$), yielding an overall specificity of 100%, a PPV of 100%, an NPV of 87.5% and an accuracy of 95.5% in the diagnosis of bladder cancer. Notably, hTERT expression was as sensitive when detecting superficial and low grade tumors as when detecting invasive high grade ones [sensitivity 66.7, 90 and 100% for tumors pTa, pT1 and pT2-4, respectively ($P>0.05$), and 83.3, 92.3 and 100% for tumor grades I, II and III, respectively ($P>0.05$)]. No significant difference was observed in the sensitivity of the test in detecting different tumor types (100, 85.7 and 92.9% for Bilharzial SCC, Bilharzial TCC and non-Bilharzial TCC, respectively; $P>0.05$). The estimated cut-off between non-neoplastic and neoplastic cases was >0.57, the cut-off between superficial and invasive tumors ≥3.7, and that between low and high grades >4.2 (Fig. 1).

Detection of chromosomal aberration in urine sedimented cells. Cells collected from the second morning urine samples were fixed on slides and examined by M-FISH for the presence of chromosome 3, 9, 7 and 17 aberrations. The slides were considered FISH-positive for bladder cancer if a gain of ≥2 chromosomes (3, 7 and/or 17) was present in the same cell, or if ≥12 of the 25 examined cells showed homozygous deletion of 9p21 (Mayo Cytogenetics Laboratory protocol, 2002). All control subjects were free of chromosomal aberrations. Of the benign cases, 3 (20%) demonstrated 9p21 deletion. Notably, they were the same cases which showed
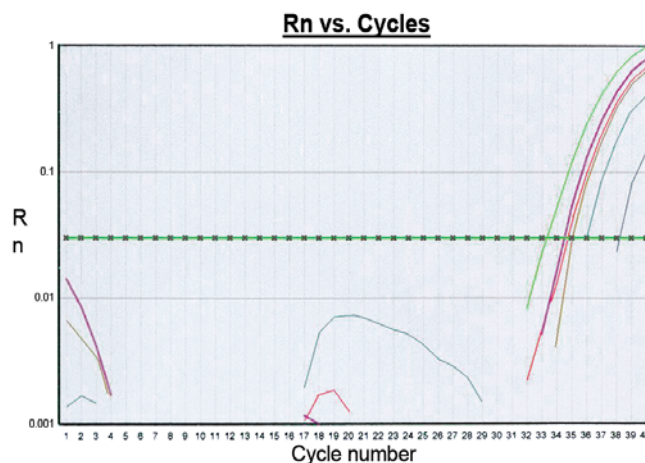

Figure 1. Real-time PCR amplification blot for hTERT mRNA in bladder cancer patients.

minimal expression of hTERT. Among the 30 cases of bladder cancer, 27 (90%) demonstrated chromosomal abnormalities. The specificity of FISH for the detection of bladder cancer was 85.7%, the PPV 93.1%, the NPV 80% and the accuracy 88.6%. FISH was significantly less sensitive when detecting superficial and low grade tumors [sensitivity 33.3, 90 and 100% for pTa, pT1 and pT2-4, respectively ($P<0.05$) for pT2-4 and pT1 compared to pTa, and 50, 100 and 100% for tumor grades I, II and III, respectively ($P<0.05$) for grades II and III compared to grade I]. No significant difference in sensitivity to different pathological tumor types (100, 85.7 and 85.7% for Bilharzial SCC, Bilharzial TCC and non-Bilharzial TCC, respectively) was observed.

Utility of urine markers in the detection and prediction of bladder cancer recurrence. As previously mentioned, follow-up was performed for 12 months. Only 20 patients could be followed after the start of therapy. In 10 of these, recurrence with a higher stage or grade tumor was detected within 4-12 months.

In terms of cytological examination and hematuria screening, 12 cases presented as positive during follow-up. Only 4 of these were histopathologically confirmed as tumor recurrence. Of the 8 urine cytology and hematuria-negative patients, 6 developed recurrence. The FDP test recorded positive results in 10 of the 20 follow-up patients, only 4 of which were histopathologically proven to be true positive. Six of the 10 FDP-negative patients demonstrated tumor recurrence. During follow-up, hTERT was detected in 8 patients, of which only 4 were confirmed as true positive. Of the 12 hTERT-negative patients followed, 6 developed recurrence. Despite a lack of significance, the BTA test was more sensitive and more specific in detecting recurrence than the other tests. It recorded positive results in 12 of the 20 follow-up patients, 8 of which were histopathologically confirmed as true positive. Of the 8 BTA-negative patients, only 2 developed recurrence. Detection of chromosomal aberration using M-FISH (Fig. 2) was the most sensitive and specific test for detecting tumor recurrence as compared to the other urine markers studied, exhibiting statistically significant utility for the prediction of
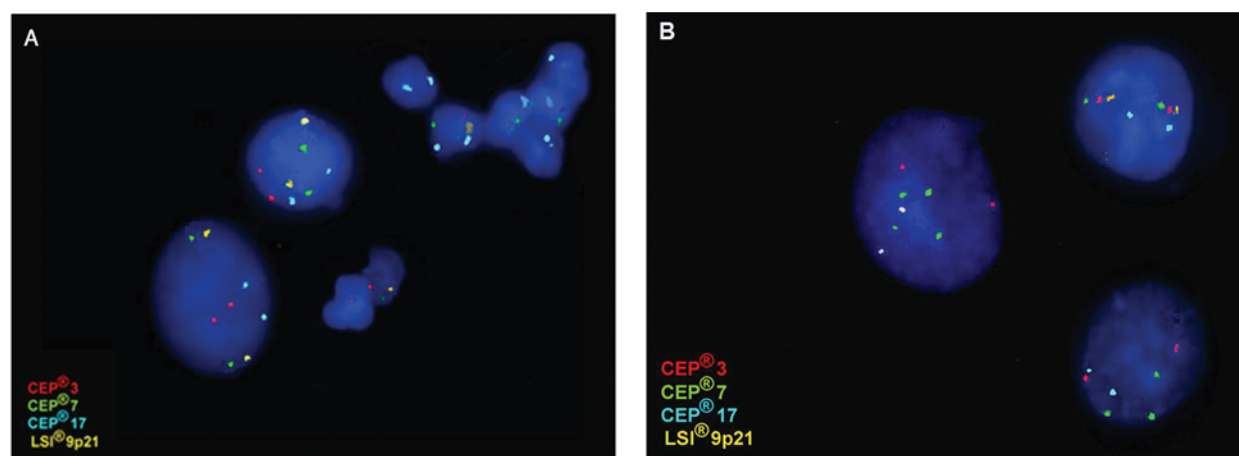
Figure 2. M-FISH. (A) A chronic cystitis case with a duplicate number for all signals. (B) A bladder cancer case. Cells with polysomy 7 are marked in green and homozygous deletion of 9p21 is in yellow.

Table III. Significance of urine markers in the detection of bladder cancer recurrence.

	Cytology	Hb Dipstick	BTA stat	FDP	Normalized hTERT	M-FISH
True positive	4	4	8	4	4	10
False positive	8	8	4	6	4	2
P-value	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
True negative	2	2	6	4	6	8
False negative	6	6	2	6	6	0
P-value	>0.05	>0.05	>0.05	>0.05	>0.05	<0.05
Sensitivity (%)	40	40	80	40	40	100
Specificity (%)	20	20	60	40	60	80
PPV (%)	33.3	33.3	66.7	40	50	83.3
NPV (%)	25	25	75	40	50	100
Accuracy (%)	30	30	70	40	50	90

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

recurrence. Of the 20 follow-up patients, 12 recorded as FISH-positive. Ten of these were histopathologically confirmed. None of the 8 FISH-negative patients developed recurrence. The significance of the urine markers studied for the detection of bladder cancer recurrence is summarized in Table III.

Statistical analysis. Analysis of the data was performed using SPSS version 11 software. The unpaired Student's t-test was used to test the significance between the quantitative data of two groups. ANOVA was used to compare quantitative data among different groups. The list significant difference test was used to compare quantitative data between two groups if the ANOVA was significant. The comparison of non-parametric quantitative data in two different groups using their mean rank was performed using the Mann-Whitney test. The χ^2 test was used for the comparison of qualitative variables in two or more groups. Receiver-operating characteristic curves were used to calculate the specificity, sensitivity and accuracy of cut-off value between two groups (18).

Discussion

At present, the standard procedure for the early detection of bladder cancer includes cystoscopy and urine cytology. The high rate of recurrence of these tumors, together with the frequent equivocal results obtained by urine cytology and the limited utility of cytology for detecting low stage and grade bladder cancer, implies that regular invasive cystoscopy is still required to diagnose and monitor patients for recurrence and progression. Non-invasive diagnostic tools that overcome these limitations would be of great import (19). Various alternative laboratory tests based on the detection of different substances, such as BTA, FDP and hyaluronic acid, have become available for the diagnosis of bladder cancer, in an attempt to improve on the diagnostic accuracy of cytology. However, the low in comparison with urine cytology sensitivity and unsatisfactory specificity reported have limited their clinical utility; none have proven capable of replacing urine cytology in the management of bladder cancer (20). Recent

studies on urine markers reported that the evaluation of hTERT expression and chromosomal aberrations would be more useful for early bladder cancer detection (21,22). In the present study, the performance of non-invasive quantitative measurement of hTERT expression and of the FISH assay in urine for the detection of different stages and grades of bladder cancer was evaluated in schistosome-related and non-schistosome-related bladder cancers, and compared to the performance of conventional urine cytology and other urine markers.

Bilharzia-associated bladder cancer represented 53.3% of all the cases studied, a finding which was expected due to the high prevalence of Bilharzial infection (a risk factor for bladder cancer) among Egyptians as reported in many studies (23). However, unlike most previous studies, TCC represented 70% of the bladder cancers studied, and the prevalence of Bilharzial TCC (43.2%) was almost equal to that of Bilharzial SCC (56.8%). In most other studies in Egypt, SCC was the more common type of bladder cancer, representing about two thirds of Bilharzial cases (23,24). Supporting our results, NCI, Cairo has reported that the pattern of bladder cancer in Egypt began changing in 1993, and that by 2005 TCC represented 62.8% of all recorded cases. The rising incidence of TCC in Bilharzia-associated bladder cancer could be due to appearance of new environmental risk factors among Egyptians. This calls for further study (25).

The sensitivities obtained by cytology in the present study were quite similar to the sensitivity ranges reported in the literature (11,26), with good sensitivity in detecting high stage and grade tumors, but insufficient sensitivity in the case of superficial and well-differentiated tumors. This could be attributed to the fact that it is very hard to distinguish well-differentiated tumors from reactive urothelial changes in urine samples (6). In urine cytology, hematuria screening was more sensitive to high stage and grade tumors, but showed very poor specificity for superficial and low grade tumors. Similar results were reported by Halling *et al* (11). Hematuria has low specificity because it is a common finding in both malignant and non-malignant genitourinary disorders, such as stones and infections.

BTA, a human complement factor H-related protein (hCFHrp), is produced by cancer cells. It interacts with complement factor C3b to inhibit the formation of the membrane attack complex, preventing cancer cell lysis and allowing the invasion of the host immune system (27). Although the BTA test showed better overall sensitivity than cytology for detecting bladder cancer, it had lower specificity with a high false-positive rate. This could be related to hematuria, as all false-positive BTA cases were associated with positive Hemastix tests. Similar observations were reported by Halling *et al*, who explained that the false-positive of BTA was associated with hematuria due to the fact that the plasma human complement factor H protein could be recognized by the antibodies of the BTA test (11). In the present study, the FDP test had similar overall sensitivity to BTA but a higher specificity, though still lower than that of cytology. Increased angiogenic factors in malignancy lead to an increase in vascular permeability and cause the leakage of plasma protein, including plasminogen, fibrinogen and other clotting factors, in extravascular tissue. Clotting factors convert fibrinogen into an extravascular fibrin clot, which is degraded by plasmin into FDP. However, various

non-specific urinary inflammatory conditions can also result in detectable amounts of FDP in the urine (28). As is the case with most of urine markers, the sensitivity of BTA and FDP declined significantly with decreasing grade and stage, with no difference between pathological tumor types. Several previous studies support our findings (11,29). In contrast, Khaled *et al* reported very high sensitivity (99%) for BTA in detecting Bilharzia-associated bladder cancer (27). Moreover, Oeda and Manabe reported high sensitivity for the FDP test in detecting low grade (70%) and non-invasive (75%) tumors, and recommended FDP as a marker for bladder cancer screening. The use of the sensitive AuraTek immunoassay in their study may be responsible for this disagreement (30).

Studies on the activation and/or expression of human telomerase in urine have generated promising results regarding its use as a non-invasive surveillance tool for early bladder cancer detection. In the present study, hTERT expression was demonstrated in 93.3% of bladder cancers, and recorded the highest sensitivity for detecting superficial (66.7%) and low grade (83.3%) ones. It should be noted that similar or occasionally better rates have been reported by other study groups using similar and different methods, such as the telomeric repeat amplification protocol (TRAP) assay (31,32). Moreover, Breslow *et al* reported that increased telomerase activity is associated with the acquisition of malignancy, and may have potential as a biomarker for the early detection of cancer (33).

Our study showed minimal hTERT expression in four benign cases; we therefore established a cut-off value >0.57 ng/μl for bladder cancer diagnosis. One of these cases had suspicious cytology and another was M-FISH-positive for bladder cancer. The detection of hTERT expression in these benign lesions could be due to presence of cells with malignant potential, prior to development of morphologic changes. Alternatively, the lesions may have a small subset of malignant cells at the time of molecular investigation, or the lesions could be infiltrated by activated lymphocytes, which have been documented to show transient telomerase activity (31). Similar results were reported by Bialkowska-Hobrzanska *et al* (21). All their benign cases expressing hTERT developed cancer within 3-6 months, confirming the accuracy of molecular diagnosis. Another study has described positive molecular tests before cancer detection by conventional diagnostic systems (34). Moreover, Rehat *et al* reported telomerase activity in histopathologically-dysplastic tissues taken from TCC patients near the area of resected tumors (35). They interpreted the presence of telomerase activity as an indicator of the onset of the process of carcinogenesis in the tissue located at the periphery of the cancer field. In contrast to previous reports, none of the healthy volunteers in our study were found positive for hTERT expression (21,32,36). Our findings are supported by the results of Melissourgos *et al* (31). The discrepancies between the different studies could be attributed to the existing variability in the sample selection. For example, Ito and colleagues had analyzed normal tissues lying adjacent to cancerous lesions, which occasionally carry the risk of incomplete tumor cell exclusion.

Recently, several assays have been developed for bladder cancer diagnosis using FISH technology, based on the detection of chromosomal aberrations present in exfoliated urothelial

cells obtained from urine or bladder irrigation specimens. FISH has thus been regarded as a potential tool for the diagnosis of bladder cancer and the prediction of recurrence (20). In the current study, M-FISH increased the sensitivity rates obtained by urine cytology for bladder cancer detection across all histological grades and stages, with no significant difference in specificity. FISH shows its greatest potential in comparison to cytology when applied to superficial and low grade tumors, in which the appearance of the exfoliated tumor cells resembles normal urothelial cells, making cytology of little use in diagnosis (33.3 vs. 0% for pTa tumor stage and 50 vs. 16.7% for grade I tumors). Our results are supported by the studies of Halling *et al* and Placer *et al* (11,20). On the other hand, M-FISH was markedly less sensitive than hTERT expression for the detection of superficial tumors at the pTa stage (33.3 vs. 66.7%). Halling *et al* reported that small urine sample volume or small-size tumors in which cells do not readily exfoliate may explain the low sensitivity of M-FISH in the case of pTa tumors (11). Moreover, previous studies have shown that more than 90% of low grade pTa tumors are diploid, with only a few showing chromosomal alterations (1).

In the present study, the homozygous deletion of 9p21 represented the most common chromosomal aberration in all cases collectively (63% of cases). It was the only aberration detected in pTa tumors (33.3% of cases), and the most common one in grade I cases (66.6% deletion of 9p21 vs. 16.7% polysomy 3), suggesting that 9p21 deletion is one of the early events in bladder carcinogenesis. Similar results have been reported by Halling *et al* (11). On the other hand, polysomy 7 and polysomy 17 were mainly associated with advanced and high grade tumors. Similarly, previous studies have reported an association between polysomy 7 and 17 and highly aggressive tumor types (6,37). One of the important target genes in polysomy 7 is monkey erythroblastosis oncogene-B (c-erbB)1, which codes for epidermal growth factor receptor (EGFR). Overexpression of EGFR has been strongly correlated with high stage and grade bladder cancer (38). Similarly, chromosome 17 harbors HER-2neu proto-oncogene, a gene which regulates the phosphorylation of B-catenin, leading to the inactivation of E-cadherin mediated cell adhesion and favoring invasive properties and metastatic potential (39).

In the present study, M-FISH was the most sensitive marker for the detection of tumor recurrence (100 vs. 40% for all other studied markers), suggesting that M-FISH could be used as a valuable predictor of tumor recurrence. Our results are supported by the study of Bubendorf *et al*, who reported that patients with M-FISH-negative urine are at a low risk of early recurrence and might be candidates for a reduced frequency of follow-up cytology (6).

In conclusion, the assessment of hTERT mRNA and chromosomal aberration (M-FISH) in exfoliated urothelium represents a highly sensitive tool for the early detection of bladder cancer. It seems conceivable that these emerging urine-based molecular assays could progressively replace urine cytology and form a non-invasive reliable adjunct to cyctoscopy for early bladder cancer diagnosis. The high sensitivity of FISH in the detection of recurrence suggests the use of FISH in combination with urine cytology as an alternative of cyctoscopy during follow-up.

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