

P27 and mdm2 as molecular grading biomarkers in transitional cell carcinoma

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ABSTRACT

To investigate the clinical relevance of p27 and mdm2 gene expression in transitional cell carcinoma (TCC), urine samples were collected from 30 TCC patients (preoperatively), 10 patients with nonmalignant urological diseases and 10 controls. Total RNA was extracted from urine sediments and RT-PCR was performed. Absence of p27 mRNA and mdm2 acquisition was specific for grade III TCC. Recurrences significantly increased with schistosomiasis and larger tumor size. Overall survival significantly shortened with larger tumor size, tumor multiplicity, and solid tumor growth pattern. In multivariate analysis, the tumor growth pattern was significant prognostic factor ($p=0.036$) for disease-free and overall survival while, high grade (exactly significant) and solid tumor growth pattern ($p=0.023$) were significant prognostic factors for the p27-/mdm2+ genetic pattern in TCC. We conclude that efficient management of TCC would be enhanced using p27 or mdm2 mRNAs as molecular grading markers. [Turk J Cancer 2008;38(2):68-77]

KEY WORDS:

Transitional cell carcinoma, molecular markers, grading, exfoliated cells

INTRODUCTION

Bladder cancer is an important cancer type in Egypt related to high prevalence of schistosomiasis. It accounts for 32.7% of all diagnosed cancers and it ranks the first among male cancer sites. Bladder cancer is characterized by gender differential incidences and a recent change in the histological type (1,2).

Bladder tumors of similar histological grade and stage have variable behavior, suggesting that genetic alterations must be present to explain the diverse behavior of bladder cancer. Current grading system is insufficient to accurately predict the evolution of most invasive bladder cancers irrespective of treatment. A need for molecular tumor markers that could be incorporated into clinical practice to add prognostic information to the conventional grading system in terms of treatment response and prognosis is crucial (3).

Transformation of a normal urothelial cell into malignant one is a sequential complex process during which expression of different classes of genes including cell-cycle genes is altered. Bladder genetic alterations are believed to result in two different types of tumor behavior (papillary growth or carcinoma in situ and subsequent invasion) with differential frequency of deletions in chromosome 9 and various percentages of p53 mutations. As the disease progresses into more aggressive and metastatic form, more various alterations of several other chromosomes take place (3,4).

P27 (Kip1) is a cell-cycle regulator involved in growth inhibition, drug resistance, apoptosis, differentiation, inflammation, actin dynamics and cell migration (5,6). Human p27 gene is localized on chromosome 12p12-13. The gene contains two coding exons and non-coding one. The cDNA encodes a phosphoprotein of 198 amino acids (7). A large number of studies have characterized p27 as a prognostic factor in various human cancers, including bladder cancer. Overexpression of p27 was detected in low-grade, superficial, papillary and slowly proliferating TCCs (transitional cell carcinomas) (8-10). Conversely, down regulation of p27 was associated with higher tumor grade, stage, and short survival (8,9,11). Loss of p27 protein was reported to be an independent predictor of early recurrence (12).

Human mdm2 (human homologue of mdm2) is a proto-oncogene which has an extremely complex pattern of expression. Its multiple-sized transcripts and proteins have been found in tumor specimens and cell lines by several investigators (13-16). Human mdm2 gene has been localized to chromosome 12q13-14 (17,18). The gene is composed of at least 12 exons. Two p53 responsive elements were detected in intron I. Two promoters with differential dependency on p53 were detected upstream of exon I and II. Up to seven alternatively sized transcripts of the human mdm2 were found in various human tumors including bladder tumors (18-20). The expression of the alternatively sized forms was found to be more frequent in tumors of advanced stage and high histological grade, and they also retained their transformation ability (14,18). Gene amplification and overexpression of mRNA were both detected in bladder cancer where they are correlated with poor prognosis (19-21).

SUBJECTS AND METHODS

Subjects

This study included 50 participants from patients followed up in the department of Urology of the University of Tanta (Egypt), subdivided as follows:

1. Thirty (70% infected with schistosoma hematobium with or without chronic cystitis and 20% actively smoking) transitional cell carcinoma (TCC) bladder cancer pa-

tients. The age ranged from 30 to 76 with a median age of 60.5 years. The male to female ratio was 9:1 (Table 1).

2. Ten (male to female ratio 1:1) patients (50-75 years) with non-malignant urological diseases (30% urethritis, 10% cystitis, 10% cystitis plus schistosomiasis, 20% hyperoxaluria, and 30% renal stones plus schistosomiasis).

3. Ten healthy volunteers (32-67 years) with male to female ratio 1:1 as control group.

Patients' samples were collected according to the ethical principles stated in the Belmont report (22).

Treatment and follow up

Treatment

The treatment of bladder cancer depends on how deep the tumor invades into the bladder wall.

Superficial bladder cancer (13/30; 43.3%)

Early bladder tumors were surgically removed by transurethral resection (TUR). Immunotherapy in the form of BCG (Bacille Calmette-Guérin) instillation was also used to treat and prevent the recurrence of superficial tumors. Patients (8/13; 61.5%) received 1 cycle BCG (once a week for 6 weeks; if necessary, this schedule may be repeated once) and regularly examined by cystoscopy for recurrence. Instillation of chemotherapy into the bladder was also used to treat superficial disease in five cases (5/13; 38.5%).

Muscle invasive bladder cancer (17/30; 56.7%)

Tumors that infiltrate the bladder required more radical surgery (7/17; 41.1%) where part or the entire bladder was removed (a cystectomy). A combination of radiation and chemotherapy as neoadjuvant and/or adjuvant therapy were also used (8/17; 47.1%) to treat invasive disease according to the medical status and patient's age. Chemotherapy was usually composed of 2-4 cycles of gemcitabine-cisplatin (day I: Gemcitabine 1250 mg/m²/day and cisplatin 70 mg/m²/day; day 8: Gemcitabine 1250 mg/m²/day; repeated every 21 days for 2-4 cycles according to the clinical status of the patient) and loco-regional radiotherapy includes 4000-6000 cG/5 weeks (5 fractions/week). Two cases had received BCG treatment (2/17; 11.8%).

Table 1
The clinico-pathological characteristics of TCC bladder cancer patients

Parameter	Bilharzial (n=21) n (%)	Non-bilharzial (n=9) n (%)	Total (n=30) n (%)
Age (years)			
Range	30-75	55-76	30-76
Mean±SD	56±14.3	62±7.1	57.5±13.1
< 60	10 (48)	5 (56)	15 (50)
≥ 60	11 (52)	4 (44)	15 (50)
Gender			
Male	18 (86)	9 (100)	27 (90)
Female	3 (14)	0 (0)	3 (10)
Male/Female ratio	6:1	–	9:1
Number of exfoliated cells (10 ⁴ cells/ml of urine)			
Range	8-57	9.8-59	8-59
Mean±SD	29±16.27	24.6±13.5	25.96±14.28
Low (8-15)	4 (19)	2 (22)	6 (20)
Moderate (16-25)	11 (52)	2 (22)	13 (43)
High (26-59)	6 (29)	5 (56)	11 (37)
RNA (10 ² µg/ml of urine)			
Range	0.76-6.64	0.9-6.7	7.6-67
Mean±SD	3.3±1.75	4±1.77	36±18
Hb (g/dl)			
≤10	12 (57)	6 (67)	18 (60)
>10	9 (43)	3 (33)	12 (40)
Histological grade			
II	11 (52)	6 (67)	17 (57)
III	10 (48)	3 (33)	13 (43)
Stage			
Ta	1 (5)	5 (56)	6 (20)
T1	6 (28)	1 (11)	7 (23)
T2a	2 (10)	0	2 (7)
T2b	7 (33)	3 (33)	10 (33)
T3a	2 (10)	0	2 (7)
T3b	3 (14)	0	3 (10)
Histological type			
Superficial	7 (33)	6 (67)	13 (57)
Invasive	14 (67)	3 (33)	17 (43)
Number of tumors			
≤3	9 (43)	6 (67)	15 (50)
>3	12 (57)	3 (33)	15 (50)

Table 1
The clinico-pathological characteristics of TCC bladder cancer patients (continued)

Parameter	Bilharzial (n=21) n (%)	Non-bilharzial (n=9) n (%)	Total (n=30) n (%)
Tumor size (cm)			
≥3	20 (95)	7 (78)	27 (90)
<3	1 (5)	2 (22)	3 (10)
Growth pattern			
Papillary	8 (38)	2 (22)	10 (33)
Solid	13 (62)	7 (78)	20 (67)
P27+/Mdm2- (good pattern)	10 (48)	7 (78)	17 (57)
P27-/Mdm2+ (bad pattern)	11 (52)	2 (22)	13 (43)
Outcome			
Local recurrence	9 (43)	3 (33)	12 (40)
Free	7 (33)	3 (33)	10 (33)
Incidence of death	5 (24)	3 (33)	8 (27)
Treatment			
Immunotherapy (BCG)	6 (28)	4 (45)	10 (33)
Radiotherapy and/or chemotherapy	10 (48)	3 (33)	13 (43)
Radical cystectomy	5 (24)	2 (22)	7 (24)

Table 2
Distribution of exfoliated cells and RNA among the studied groups

Group	Exfoliated cells (x10 ⁴ cells/ml urine)	RNA (x10 ² µg/ ml urine)
Healthy controls		
Range	2-10	3.38-6
Mean±SD	4.42±2.39	4±1
Benign urologic diseases		
Range	4.5-20	7.6-23
Mean±SD	10.86±5.83	13±7
TCC bladder cancer		
Range	8-59	7.6-67.04
Mean±SD	25.96±14.28	36±18

Follow up

Patients were followed clinically for 49 months (from 2003 to 2007). In absence of recurrence, patients were followed with cystoscopy at 3-months intervals during the first year and at 6 months intervals during the subsequent years. Local progression was documented by biopsy and computed tomography (CT) scan.

Exfoliated cells

Voided urine samples (50 ml) were collected under aseptic conditions. Exfoliated cells were separated by centrifugation at 3000 rpm for 10 minutes, re-suspended in 1X PBS (phosphate buffered saline) (Ambion, USA), and counted by hemocytometer (Marienfeld, Germany).

Urine cytology

Following a 20 minutes storage period at 4°C, cells were submitted to a second 10 min centrifugation and fixed in a fixative solution (70% ethanol). The fixative

Table 3
Unfavorable prognostic factors for bad genetic profile in TCC

Variable	n	Good Pattern (n=17)	P-value	Bad Pattern (n=13)	P-value
Schistosomal infection					
Positive	21	11 (52.3)	0.225	10 (47.6)	0.052
Negative	9	6 (66.6)		3 (33.3)	
Grade					
II	17	17 (100)	p <0.001	–	p <0.001
III	13	–		13 (100)	
Tumor size (cm)					
≥3	27	15 (88)	0.002	12 (92.3)	0.002
<3	3	2 (12)		1 (7.7)	
Growth pattern					
Solid	20	8 (47)	0.808	12 (92.3)	0.002
Papillary	10	9 (53)		1 (7.7)	

Table 4
Survival of patients with TCC

Variable	n	Recurrence (n=12)	P-value	Death (n=8)	P-value
Schistosomal infection					
Positive	21	10 (47.6)	0.021	5 (23.8)	0.480
Negative	9	2 (22.2)		3 (33.3)	
Number of tumors					
>3	15	7 (46.6)	0.564	7 (46.6)	0.034
≤3	15	5 (33.3)		1 (6.6)	
Tumor size (cm)					
≥3	27	11 (40.7)	0.004	8 (29.6)	p <0.001
<3	3	1 (33.3)		–	
Growth pattern					
Solid	20	5 (25)	0.564	8 (40)	p <0.001
Papillary	10	7 (70)		–	

solution was partially but not completely removed, the pellet was resuspended, dropped onto pre-cleaned microscope slides and dried for 24 hours at room temperature. Slides were first examined under low magnification to determine the quality of the slides and the presence of inflammatory signs (bacteria, leukocytes as irritants of the bladder). Slides were then stained in hematoxylin for 4 minutes, washed with tap water, and stained with eosin

for 2 minutes. Samples were passed in ascending series 70%, 80%, 95%, absolute ethanol for 1 minute. Clearing was performed in equal parts of absolute ethanol and xylene mixture followed by two subsequent changes in xylene only. Slides were finally mounted by adding one drop of Dpx Mountant (Chematec, U.K) and examined by microscope ZEISS (Axioskop2, Germany). Stained urinary bladder cells were counted in all slides and photographed.

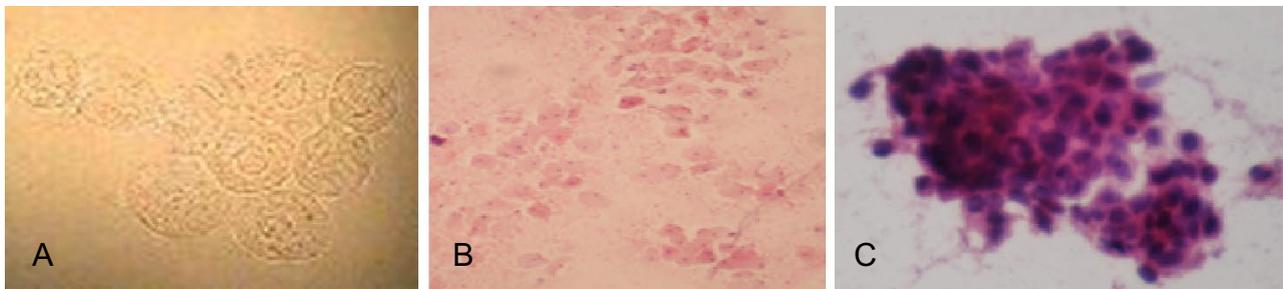


Fig 1(A,B,C). Urine Cytology. (A): unstained normal transitional cells of the bladder, (B): normal transitional cells of the bladder stained by H&E, (C): malignant transitional cells of the Bladder stained by H&E

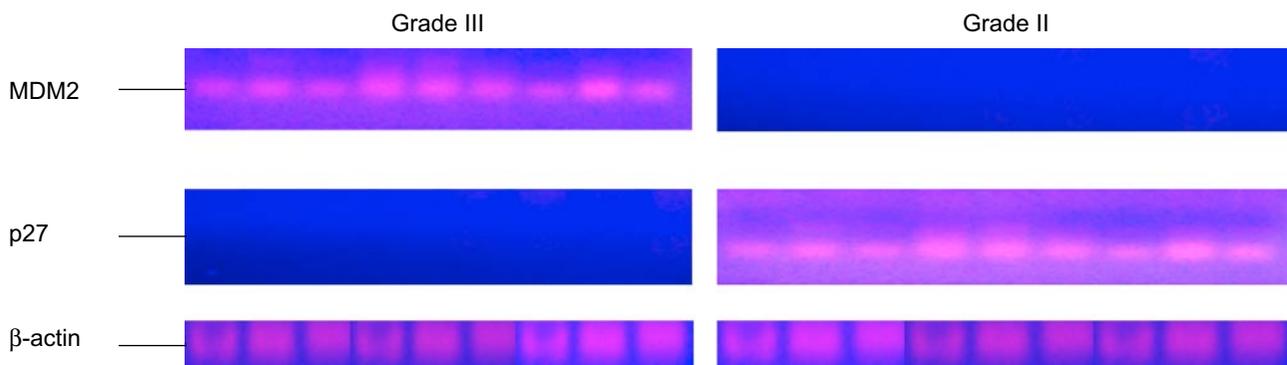


Fig 2. Gene expression in patients with TCC. Exfoliated cells were isolated from urine samples. RNA was extracted, reverse transcribed, and PCR amplified using MDM2 or P27 or b-actin specific primers. Amplified 310, 241, and 154 bp bands of MDM2, P27, and b-actin, respectively were separated on 3% agarose gel containing ethidium bromide and visualized by UV

RNA extraction

Total RNA was extracted from exfoliated cells according to the protocol of Trizol reagent (Invitrogen). RNA concentration and purity were detected spectrophotometrically while integrity was examined by gel electrophoresis.

RT-PCR

Total RNA was reverse transcribed and amplified using ready-to-go RT-PCR beads (Amersham) according to the manufacturer instructions. The following primers were used to amplify 310, 241, and 154 base pairs of mdm2 (NM_002392; from 486 to 796), p27 (NM_004064; from 585 to 826), and β -actin (X00351; from 781 to 935), respectively.

Amplification protocol has an initial denaturation for 2 minutes at 94°C, 45 cycles: 1 minute at 94°C, 90 seconds at 46°C for mdm2 and p27 or 60°C for β -actin, and 1 minute at 72°C, and a final elongation for 10 minutes at 72°C. The amplified products were separated on 2% agarose gel containing ethidium bromide and visualized by UV. Negative controls were included in each RT-PCR

experiment to ensure absence of contaminants. Amplified and non-amplified controls were also included.

Statistical analysis

Results were analyzed using the SPSS software package version 12. Differences between groups were examined using the χ^2 and exact tests. The results are expressed as percentage, mean±standard deviation, and median. Univariate and multivariate analyses of covariance were performed and a significance level of 0.05 was chosen.

RESULTS

Invasive histological type

A significant association was observed between invasive histological type and schistosomal infection (p=0.008), tumor size (p <0.001), and tumor growth pattern (p=0.029).

Exfoliated cells

Urothelial epithelial cells were detected in all of the studied groups (Table 2, Figure 1A). Hematoxylin and

eosin (H&E) staining was used to differentiate between normal and malignant transitional cells of the bladder (Figures 1B&C). No malignant cells were detected in the controls or patients with benign urological diseases. The ratio of normal to malignant bladder transitional exfoliated cells in the TCC group was 1 to 5. No significant difference in the count of exfoliated bladder cells was observed between bilharzial and non-bilharzial TCC subgroups. Large difference was observed between male- and female-urine samples. Females had a higher number of total epithelial cells mainly of vaginal origin.

Molecular studies

The presence, integrity and equal loading of RNA samples were examined using β -actin as a reference gene. β -actin was expressed in all of the examined samples in the studied groups (Figure 2). Mdm2 mRNA was absent in controls and patients with benign urologic diseases. Mdm2 mRNA remained absent in grade II bladder cancer group. Acquisition of mdm2 expression was detected in grade III of TCC bladder cancer patients (Figure 2). P27 mRNA was detected in controls and patients with benign urologic diseases. The gene expression of p27 remained detectable in grade II bladder cancer. Loss of p27 expression was the main characteristic of grade III TCC bladder cancer (Figure 2).

In univariate analysis, p27+/mdm2- genetic pattern (good) was significantly associated with grade II and tumor size ≥ 3 cm while, p27-/mdm2+ genetic pattern (bad) was significantly associated with schistosomal infection, grade III, tumor size ≥ 3 cm, and solid tumor growth pattern (Table 3). In multivariate analysis, grade III ($p < 0.001$) and solid tumor growth pattern [relative risk (95% CI): 13.5 (1.4-128.25); $p = 0.023$] were significant prognostic factors for the bad genetic profile in patients with TCC bladder cancer.

Clinical outcome

Recurrence and death rates were 40% (12/30) and 26.7% (8/30) among patients with TCC bladder cancer. In univariate analysis, schistosomal infection and tumor size ≥ 3 cm were significant prognostic factors for disease-free survival while, tumor number ≤ 3 , tumor size ≥ 3 cm, and solid tumor growth pattern were significant prognostic factors for overall survival (Table 4).

In multivariate analysis, the tumor growth pattern was a significant prognostic factor [relative risk (95% CI): 0.079 (0.007-0.843); $p = 0.036$] for disease-free and overall survival.

DISCUSSION

This study assesses; for the first time; the added value of p27 and mdm2 in bladder cancer. The results revealed a contrary pattern of expression of p27 and mdm2, therefore either of them is sufficient in molecular grading of TCC. Using such a simple non-invasive molecular test offers better identification of tumors likely to progress to muscle invasive disease and thus facilitates effective management of the disease.

Exfoliated cells

Exfoliated cells were detected in controls [Range: 2-10 (10^4); $M \pm SD$: 4.42 ± 2.39 (10^4) cells/ml urine]. Normally, the epithelial cells lining the luminal surface of the bladder slough off the surface with an extremely slow turnover rate (every 1-3 weeks) (23,24). Therefore, the presence of exfoliated cells in controls reflects the turn over rate of the normal urinary epithelial cells.

Exfoliation rate increased significantly (2.5 fold $p = 0.002$) in patients with urological benign diseases than controls. This might be explained by the fact that the urological benign diseases group in the current study included different stimuli of cellular stress (bacterial and/or viral infection, schistosomiasis, renal stones). It is evident that moderate stress elicits an enhanced desquamation of superficial cells via local detachment of tight junctions, loss of the permeability barrier, and disjunction of desmosomes accompanied by exocytosis of lysosomal enzymes in the intercellular space (25). In addition, exfoliation represents a normal host defense response against urinary tract infections (UTIs) (26-28).

Exfoliation rate in TCC group increased significantly (5.9 fold; $p < 0.001$) than controls and (2.4 fold; $p < 0.001$) than urological benign diseases group. Exfoliation and desquamation as part of the repertoire of urinary tissues are retained by their tumors (29,30). Epithelial tissues are in immediate contact with genotoxins and excreted metabolites in urine. The number of malignant cells detected in our study was 5 times greater than normal epithelial

cells as indicated by the H&E staining. Uniform cells are more likely to be normal or may be associated with hyperplasia (e.g., some cases of polypoid cystitis). Squamous epithelial cells are also commonly seen in urine and come from the terminal urethra, vagina, vulva, and preputial epithelium and they account for the sexual difference in the number of exfoliated cells in the urine sediments.

RNA

RNA extracted from exfoliated cells was also increased significantly (3.3 fold; $p=0.0002$) in patients with urological benign diseases and 9 folds in TCC group ($p < 0.001$) than controls. There was also a significant 2.8 fold increase in RNA amount in TCC than patients with urological benign diseases ($p < 0.001$). Several studies detected a differential increase in the level of mRNA (CK20, Ki-67, CD44, telomerase, c-erbB-2 and hCG- β) in exfoliated cells in bladder tumors than controls or patients with urinary infections (31-36). Therefore, our data might reflect a transcriptional activation response to the various stimuli in both urological benign diseases and TCC groups.

Molecular studies

Because epithelial cells are derived from basal cells, recent genetic damage to the basal layer of the bladder could be reflected in the presence of exfoliated cells (37-39). No prior study was conducted on assessing the gene expression of p27 and mdm2 in urothelial exfoliated cells.

P27

In this study, the absence of p27 gene expression was significantly associated with both high tumor grade and solid tumor growth pattern. Similar association between decreased p27^{Kip1} staining and higher tumor grade has been reported (40,41). No reports are available regarding the tumor growth pattern and p27 expression level.

Although several studies had reported a significant association between the low p27 protein level and poor overall and post-relapse survival, we were unable to detect such correlation (40, 42-44). This discrepancy would be explained by difference in the sample size and detection of protein instead of mRNA.

Mdm2

Similarly the acquisition of mdm2 gene expression significantly associated with both high tumor grade and solid tumor growth pattern. Tuna et al. (45) reported that the percentage of mdm2-positive cells showed a significant relationship with tumor grade and recurrence in superficial bladder cancer. In contrary to Korkolopoulou et al. (46), our data revealed a significant association with tumor solid growth pattern perhaps due to the difference in the characteristics of patients. Interestingly, we both were unable to detect any significant relation between survival and mdm2 expression pattern. It was also reported that multiple markers in association with mdm2 are better for predicting survival (47).

Interestingly this is the first report regarding the association between absence of mdm2 and larger tumor size and schistosomal infection. These results reflect an indirect relation between tumor aggressiveness represented by the absence of mdm2 and poor prognosis represented by the larger tumor size (45,46,48). Schistosomal infection is known to induce chronic irritation and inflammation in the urinary bladder, and hence it facilitates initiation of premalignant lesions and action as a promoting agent to increase the conversion of these lesions to the malignant state. Activated macrophages at the sites of inflammation are implicated in the generation of carcinogens and reactive oxygen species that lead to DNA damage and subsequently to events such as mutations, DNA strand breaks, and sister-chromatid exchanges (49).

Clinical outcome

Tumor grade, size, multiplicity, growth pattern are well known to affect the outcome of urinary bladder carcinoma (48). Same results were obtained by the current study. Interestingly, schistosomal infection significantly affected the disease-free survival in the current study. The data would be explained by the significant relation between invasive histological type and schistosomal infection detected in this study. It is well documented that the anatomic extent of the disease is the single most important factor affecting outcome in bladder carcinoma (48). Mutations of genes associated with cell cycle control were detected in schistosomal-associated bladder cancer (49).

In conclusion, p27 or mdm2 would be useful as molecular markers in grading TCC. The potential for using cell cycle molecular markers with exfoliated urothelial

cell RNA could establish an individualized fingerprinting as a method of surveillance for both tumor recurrence and progression as an adjunct to cystoscopy and cytology.

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