

# The value of combined use of survivin mRNA and matrix metalloproteinase 2 and 9 for bladder cancer detection in voided urine

Sanaa Eissa<sup>a,\*</sup>, Soheir Badr<sup>b</sup>, Shadia Abd Elhamid<sup>c</sup>, Azza Salah Helmy<sup>c</sup>, Mohamed Nour<sup>b</sup> and Mohamed Esmat<sup>d</sup>

<sup>a</sup>Biochemistry Department, Faculty of Medicine, Ain Shams University, Cairo, Egypt

<sup>b</sup>Oncology Diagnostic Unit, Faculty of Medicine, Ain Shams University, Cairo, Egypt

<sup>c</sup>Biochemistry Department, Faculty of Science, Ain Shams University, Cairo, Egypt

<sup>d</sup>Urology Department, Faculty of Medicine, Ain Shams University, Cairo, Egypt

**Abstract.** *Objective:* In a trial to improve the diagnostic efficacy of conventional urine cytology we determine survivin RNA and matrix metalloproteinase 2 and 9 in urine of bladder cancer cases.

*Method:* Voided urine specimens were collected from patients with histologically confirmed bladder urothelial carcinoma (Group 1;  $n = 46$ ), urological patients without urothelial carcinoma (Group 2;  $n = 20$ ), and healthy volunteers (Group 3;  $n = 20$ ). Urine cytology, survivin RNA was estimated by qualitative nested RT-PCR and MMP-2, MMP-9 activity were detected by gelatin zymography. The expression of survivin RNA and matrix metalloproteinase 2 and 9 in bladder cancer was compared with benign and normal cases.

*Results:* Positivity rates of survivin RNA and MMPs zymography were significantly different among the 3 groups. Urine survivin detection by qualitative nested RT-PCR showed 76.1% sensitivity and 95% specificity. The overall sensitivity, specificity of urinary MMP zymography was 67.3%, 90% respectively. The combined use of urine cytology with urine survivin or MMPs zymography increased sensitivity of urine cytology from 50% to 84.7%. The highest sensitivity (95.6%) was obtained on combining the three markers.

*Conclusion:* Survivin RNA and MMPs zymography can be considered as promising noninvasive markers for bladder cancer early detection. Combined use of the three markers improved the sensitivity for detecting bladder cancer.

Keywords: Survivin, MMPs, RT-PCR

## 1. Introduction

Bladder carcinoma is one of the most common urologic malignancies occurring worldwide [1]. In Egypt it accounts for 30% of all cancers [2] and has been associated with many pathogenetic factors, most commonly bilharzial infestation [3]. Definitive proof of bladder cancers requires invasive cystoscopic examinations. However, this procedure is uncomfortable and labor-intensive, particularly in follow-up [4]. Although uri-

nary cytology is widely used for screening for bladder cancer, it is sometimes difficult to judge cytologic specimens, particularly for low-grade cancers [5]. Therefore new, noninvasive methods for bladder cancer detection would open new possibilities in diagnosis and monitoring [6], as well as in screening of groups at risk such as bilharzial infested patients [7–9]. An ideal test to monitor bladder cancer should be objective, noninvasive, easy to administer and interpret and have high sensitivity and specificity.

Survivin, also called Baculoviral IAP repeat-containing 5 (BIRC5), is a human gene that is part of the inhibitor of apoptosis protein (IAP) family. Survivin was found during embryonic and fetal develop-

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\*Corresponding author: Sanaa Eissa, Medical Biochemistry and Molecular Biology Department, Faculty of Medicine, Ain Shams University, Cairo, Egypt. E-mail: dr\_sanaa\_eissa@yahoo.com.

ment, was completely down-regulated and undetectable in normal adult tissue, and became prominently re-expressed in all of the most common human cancers, including cancers of the lung, colon, pancreas, prostate, and breast [10]. Acting at the interface between apoptosis and mitosis, Survivin was shown to be both a chromosomal passenger protein and to prevent programmed cell death by inhibiting the actions of the cell-death terminal effectors caspases-3 and caspases-7 [11].

The MMPs (matrix metalloproteinase) are a family of zinc-dependent endopeptidases that have been associated with the ability of tumor cells to degrade extracellular matrix components during tumor cell invasion [12]. They are secreted as latent proenzyme which requires activation through proteolytic cleavage of N-terminal prodomain and are detected in urine samples of bladder cancer patients either active or inactive [13]. MMPs, in particular interstitial collagenase (MMP-1), stromelysin-1 (MMP-3), stromelysin-3 (MMP-11), the gelatinases (MMP-2 and MMP-9) are found in enhanced amounts in tumor tissues, where they regulate tumor growth and metastasis, promoting the invasion of malignant cells [14] and obviously found to be associated with bad survival [15,16]. The aim of this study was to evaluate the expression of Survivin mRNA and Matrix Metalloproteinases activity in urine of malignant and benign bladder patients compared to normal control group and examined the usefulness of these markers for detection of bladder cancer.

## 2. Materials and methods

The study Population included 66 Egyptian patients admitted to the urological department Ain Shams Faculty of medicine. A blood sample and single voided urine sample were obtained from all patients after obtaining informed consent. A cytological test for urine sediment was done before cystoscopy. All patients underwent cystoscopy and histopathology as the reference standard to detect bladder carcinoma. Accordingly the 66 studied patients were classified into 46 with bladder cancer with a median age 61.5 years (range 26 to 83) and 20 with benign urological lesion with a median age 53 years (range 25 to 70). In bladder cancer, a total of 29 were transitional cell carcinoma and 17 were squamous cell carcinoma. Tumor were staged according to TNM classification of UICC criteria [17] and graded according to WHO criteria [18]. The study also included a group of 20 healthy volunteers with a median age 39 years (range 25 to 60) who were recruited from the laboratory staff.

### 2.1. Sample preparation

Venous blood (5 ml) and voided urine (30 to 60 ml) samples were obtained from all individuals before treatment or surgery. Each urine samples were collected into approved urine collection cups which were sealed immediately and placed on ice, then centrifuged for 15 to 20 minutes at 2,500 to 4,000 x gravity and separated into supernatant and pellet. Pellets were washed twice with phosphate buffered saline, pH 7.0. A portion of the pellets was used for cytological and microscopic examination by a cytologist. The other portion was treated with 500  $\mu$ l protease inhibitor cocktail composed of 0.5 gm/100 ml ethylene diamine tetra acetic acid-disodium dehydrate, 0.4 gm/100 ml phosphomethylsulfanyl fluoride, 1,000  $\mu$ l/100 ml benzamidine and 100  $\mu$ l/100 ml aprotinin [19] and stored at  $-80^{\circ}\text{C}$  for further processing to extract RNA for survivin detection.

### 2.2. Detection of serum schistosomiasis antibodies

Serum was used to detect schistosomiasis antibodies by the indirect hemagglutination test using the Cellognost<sup>®</sup> Schistosomiasis H kit [20].

### 2.3. RNA extraction

RNA was extracted using purescript RNA Isolation kit (Genera systems, Inc., Minneapolis, USA) [21]. Up to  $1 \times 10^7$  cells were disrupted and homogenized with the lysis buffer (citric acid, EDTA and sodium dodecylsulfate 'SDS'). One volume of 70% ethanol was added to the lysate and up to 700  $\mu$ l of sample were applied to the RNeasy Mini spin column. high quality RNA was eluted in ribonuclease- free water.

### 2.4. RT nested PCR for survivin mRNA

Total RNA (2  $\mu$ g) was subjected to first strand cDNA synthesis using Moloney murine leukemia virus RT and hexamer primer [pd (N)6] for 5 minutes at  $35^{\circ}\text{C}$  and for 60 minutes at  $43^{\circ}\text{C}$ . The RT-PCR reaction was performed using Ready-to-Go<sup>™</sup> RT-PCR Beads After heating at  $72^{\circ}\text{C}$  for 15 minutes a first amplification reaction was done with survivin primers (sense 5'-CTG CCT GGC AGC CCT TTC TCA A -3' and antisense 5'-AAT AAA CCC TGG AAG TGG TGC A-3') with initial melting at  $94^{\circ}\text{C}$  for 5 minutes, 35 cycles of denaturation at  $94^{\circ}\text{C}$  for 1 minute, annealing at  $55^{\circ}\text{C}$  for 1 minute and extension at  $72^{\circ}\text{C}$  for 2 minutes,

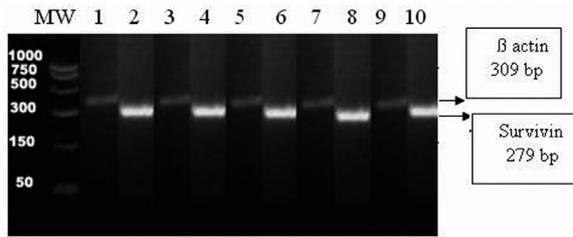


Fig. 1. RT nested PCR of urinary survivin at 279 bp and RT PCR of  $\beta$ -actin at 309 bp. Lane 1, molecular weight ladder standard at 1,000 to 50 bp. Lanes 2 to 11, bands positive for survivin and  $\beta$ -actin in bladder cancer samples.

Table 1

Survivin RNA and MMPs zymography, and urine cytology in study groups

Groups	No Pos(%) Survivin	No Pos(%) MMPs	No Pos(%) Cytology
Malignant( $n = 46$ )	35(76.1)	31(67.3)	23(50)
Benign( $n = 20$ )	2(10)	4(20)	0
Normal( $n = 20$ )	0	0	0
Chi-square*	44.5	30.8	27.3

\* $p < 0.001$ .

followed by incubation at 72C for 5 minutes. A 463 bp fragment of survivin cDNA was subjected to a second round of amplification with nested survivin primers (sense 5'-CCGCATCTCTACATTCAAGAAC-3' and antisense 5'-CTTGGCTCTTTCTCTGTCC-3') with initial melting at 95C for 5 minutes, 30 cycles at 94C for 30 seconds, 60C for 30 seconds and 72C for 1 minute, and final extension at 72C for 5 minutes. The 279 bp product for survivin was separated on 2% agarose and visualized by ethidium bromide staining [22].  $\beta$  actin was processed for use as a housekeeping gene. It's primer pair was sense 5' -AGCGGCAAATCGTGCG TG-3' and antisense 5' -CAGGGTACATGGTGCC 3' (Fig. 1).

### 2.5. MMP zymography

MMP zymography was performed according to the method of Heussen and Dowdle [23] and modified by Bianco et al. [24] 8% sodium dodecyl sulfate (SDS) mixed with gelatin (1 ml of 1 g%) was used to separate 20  $\mu$ g sample protein of urine supernatant by mini-protean II (Bio-Rad Labs, USA) electrophoresis according to the method of Sambrook et al. [25]. The gel was removed when the tracking dye front had reached the bottom of the resolving gel and then washed twice (30 min each) under constant mechanical stirring in 200 ml of 2.5% Triton X-100 at room temperature. The gel slabs were then immersed in detection buffer

Table 2

Survivin RNA and MMPs zymography, and urine cytology vs. bladder cancer group clinicopathological factors

Clinicopathological Factor	Survivin No Pos(%)	MMPs No Pos(%)	Cytology No Pos(%)
Gender			
Male (35)	27 (77.1%)	24 (68.6%)	16 (45.7%)
Female (11)	8 (72.7%)	7 (63.6%)	7 (63.3%)
Smoking			
- ve (22)	17 (77.3%)	15 (68.2%)	13 (59.1%)
+ ve (24)	18 (75%)	16 (66.7%)	10 (41.7%)
Bilharziasis			
- ve (11)	8 (72.7%)	8 (72.7%)	5 (45.5%)
+ ve (35)	27 (77.1%)	23 (65.7%)	18 (51.4%)
Pathology			
TCC (29)	21 (72.4%)	19 (65.5%)	15 (51.7%)
SCC (17)	14 (82.4%)	12 (70.6%)	8 (47.1%)
Grade			
Low grade (I + II) (36)	28 (77.7%)	22 (61.1%)	18 (50%)
High grade (III) (10)	7 (70%)	9 (90%)	5 (50%)
Stage			
Early stage (0 + 1) (15)	13 (86.7%)	9 (60%)	7 (46.7%)
Late stage (2 + 3) (31)	22 (70.9%)	22 (70.9%)	16 (66.7%)
Cytology			
- ve (23)	16 (69.6%)	16 (69.6%)	-
+ ve (23)	19 (82.6%)	15 (65.3%)	-

TCC: Transitional cell carcinoma, SCC: Squamous cell carcinoma.

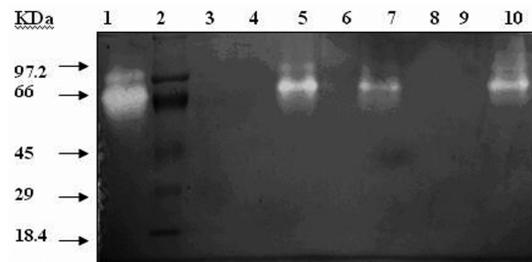


Fig. 2. Gelatin zymography for the enzymatic activity of MMP-2 and MMP-9. prestained molecular weight marker (14.7-97.2) at lane (2), Urine supernatant of bladder carcinoma. Lane1: - ve bilharziasis, SCC, grade 2, stage 1. lanes 3, 5: + ve bilharziasis, SCC, grade 2, stage 1. Lane 4: + ve bilharziasis, SCC, grade 2, stage 2 and Lane 6: + ve bilharziasis, SCC, grade 2, stage 3). Urine supernatant of benign bladder (7,8,9,10). Clear zones indicate gelatinolytic activity with molecular weights 92 KDa correspond to Pro-MMP-9, 72 KDa correspond to Pro-MMP-2 and 62KDa correspond to active isoforms of MMP-2.

and incubated at 37C for 24 h. detection buffer was added (50 mmol/l Tris-HCl (pH 7.6), 0.2 mol/l NaCl, and 5 mmol/l CaCl<sub>2</sub>).The gels were then fixed and stained by immersion for 1 h in Coomassie Blue R-250. Molecular weight protein maker appeared as easily visible staining bands against the lighter blue color of the stained gelatin background. Gelatinase activity was detected as clear zones of lyses (negative staining)

Table 3  
Combined sensitivity and specificity for survivin RNA and MMPs zymography, and urine cytology in detection of bladder cancer

Paramete	Sensitivity	Specificity	NPV	PPV	Accuracy
Cytology	50%	100%	63.45	100%	73.2%
Survivin	76.1%	95%	77.5%	94.5%	84.8%
MMP-Zymography	67.3%	90%	70.5%	88.5%	77.9%
Cytology + Survivin	84.7%	95%	84.4%	95.1%	77%
Cytology + MMP – Zymography	84.7%	90%	83.7%	90.6%	87.2%
Survivin + MMP – Zymography	91.3%	85%	89.4%	87.5%	88.3%
Cytology + Survivin + MMP – Zymography	95.6%	85%	94.4%	88%	90.6%

against the blue background. The gels were scanned to make a permanent record of the results (Fig. 2).

### 2.6. Statistical analysis

The nonparametric Mann-Whitney sum rank U test and Kruskal-Wallis ( $\chi^2$ ) test were used for the comparison of the variables between groups. Positivity rates of studied parameters were compared among malignant, benign and control groups using chi square test. The level of significance was determined to be less than 0.05. All analysis was performed with the use of the Statistical Package for the Social Sciences (SPSS Inc, Chicago, IL, USA).

### 3. Results

Qualitative RT-PCR and MMPs zymography in 46 samples revealed positivity in 76.1% and 67.4%, respectively, of the malignant group and in 10% and 20% of the benign group and not detected in normal individuals ( $p < 0.001$ , Table 1).

On correlating studied urinary markers with clinico-pathological factors of bladder cancer group, no significant relation was found (Table 2).

Sensitivity and specificity for each urinary marker as well as their combination were tested for detection of bladder cancer. Both sensitivity and specificity of urinary survivin was the highest, whereas urine cytology revealed the lowest sensitivity. Combining survivin and MMPs increased sensitivity and specificity. Sensitivity of urine cytology improved when combined with survivin RNA or MMPs zymography while the best sensitivity for urine cytology was achieved when the 3 markers were combined (Table 3).

### 4. Discussion

Soluble molecular markers secreted in urine could serve as urinary markers for bladder cancer detection,

depending on their efficiency to provide early detection capabilities and insight into appropriate treatment response and tumor recurrence [26]. Using nested RT-PCR survivin mRNA expression in urine was determined by comparing the expression of the target gene survivin with that of the housekeeping gene  $\beta$ -actin, which is thought to be suitable to normalize expression. Our results revealed a marked increase in the positivity rate of urine survivin in the malignant vs. the benign group (76.1% vs. 10%) while no normal urine samples showed positive survivin RNA ( $p < 0.001$ ). Moussa et al. [27] detected higher urine survivin RNA sensitivity (94%) and specificity (95%). Also, Smith et al. [22] reported absolute sensitivity at 95% specificity. In a recent study by Ku et al. [28], Bivariate random effect meta-analyses were used to calculate the summary estimated of sensitivity and specificity of urine surviving in 14 studies on 2051 subjects. The pooled sensitivity and specificity for urine survivin tests were 77.2% and 91.8% respectively which is nearly similar to our results. Discrepancies in urinary survivin mRNA sensitivity and specificity results may be attributable to the use of different types of samples, in which the number of living cells varies, proper transport and storage. In our laboratory we ensured the highest RNA quality by rapid sample washing, processing, preserving urine sediment in a protease inhibitor cocktail adapted at our laboratory and using a housekeeping gene to exclude samples with degraded RNA.

MMPs are important mediators of cancer progression and invasion [29]. Urinary MMPs-Zymography is one of the few urinary markers approved by the food and drug administration for monitoring and detection of bladder cancer. MMPs levels in cancer patients could in turn reflect enhanced presence of these markers in the circulation, but in the case of bladder cancer they might also directly originate from the tumor and therefore could be particularly indicative for superficial bladder tumors [26].

Our results revealed marked increase of MMP-2 and MMP-9 levels in malignant group compared with benign and control groups. In one of the few studies mea-

suring MMP-9 and MMP-2 in urine, Sier et al. [30] revealed increased level of both markers in the malignant group compared with a control group that did not include patients with benign urologic disease. This marked increase in MMPs in the malignant group is expected because MMP-2 is synthesized by tumor cells or quite commonly by host response to tumor as fibroblasts, macrophages, and vascular endothelial cells. However, MMP-9 is strongly expressed in intravascular and tissue-infiltrating leucocytes [31] and their implication in the pathogenesis of bladder cancer has been documented [30].

When a urine molecular marker is used for bladder cancer diagnostic assessment, it should have high sensitivity and PPV [27]. Accordingly urinary survivin mRNA and MMPs are significantly superior to urine cytology.

Urinary survivin levels were detected in the majority of bladder cancer cases misdiagnosed by MMPs zymography and cytology (false negative). This performance was achieved at the expense of yielding a false positive rate of 5%. Given the aggressive biological behavior of bladder cancer this minimal number of false positive results seems acceptable. In conclusion, combined use of survivin mRNA by nested RT-PCR and MMPs- zymography improve the sensitivity and specificity of urine cytology for diagnosis of bladder cancer.

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