

Value of a Combination Assay of Urinary Survivin, Calreticulin and Urokinase Type-Plasminogen Activator Receptor as Non-Invasive Diagnostic Tool in Non-Muscle-Invasive Bladder Cancer

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Abstract: Introduction: Approximately 70% of bladder cancer are superficial, and respond well to endoscopic transurethral resection. However, 70% of these patients experience tumor recurrence. As the propensity for local recurrence extends over the lifetime, these patients must undergo life-long surveillance. Combination of cystoscopy and urine cytology, is considered to be the "gold standard" for this surveillance. However, the former is invasive and the latter has low sensitivity. Therefore, new non-invasive tests with high sensitivity and specificity that are easy to perform are needed for screening and surveillance for recurrent tumors. **Aim of the Work:** The aim of this work was to investigate the value of a combination assay of the three urinary proteins: survivin, calreticulin (CRT) and urokinase type plasminogen activator receptor (uPAR) as non invasive diagnostic tool in detection of bladder cancer recurrence. **Patients and Methods:** From march 2010 to October 2010, all patients with known history of NMIBC who are scheduled for follow-up cystoscopy in the Department of Urology, Alexandria University were prospectively included in this study. All patients underwent cystoscopy under general anaesthesia, and those who were found to have a definite or suspicious lesion(s) in the bladder underwent complete TURBT. Voided urine samples from all patients were taken before cystoscopy. Urinary survivin and uPAR concentrations were performed by ELISA technique while urinary CRT was estimated by western blot technique. **Results:** Sixty eight patients were eligible to our study. Thirty patients were found to have no recurrence of the disease and were considered as group I (recurrence-free group) while 38 patients had non-muscle-invasive recurrence and were considered as group II (recurrence group). There was significant increase of the three urinary proteins in the recurrence group compared to the recurrence-free group. The concomitant use of the three urinary markers revealed higher sensitivity for detection of bladder cancer recurrence (96.8%) than the use of each marker alone, but at the expense of lower specificity (80%). Combining these markers using a Logistic Regression Model resulted in higher specificity with maintained excellent sensitivity. A direct comparison between the diagnostic performance of the new logistic regression model, survivin alone and various combinations the three markers showed that the new model had the highest sensitivity (93.75%) with a 100.00% specificity. **Conclusion:** Combining more than one urinary marker is a logic step forward that improves the sensitivity of detection of bladder cancer recurrence. The use of this logistic regression model as a promising urinary marker for early detection of bladder cancer is recommended where the specificity remains 100.0% while the sensitivity is raised to 93.75%. However larger studies should be carried out to prove the usefulness of these marker combinations.

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1. Introduction

Bladder cancer is a major health problem worldwide imposing a great medical and economic burden on the patient and his society⁽¹⁻²⁾. Seventy five to eighty percent of TCC of the bladder are non-muscle-invasive (NMIBC) at diagnosis⁽³⁾. As this category carries high risk of recurrence and / or progression, its management necessitates life long follow up to early detect recurrence and hence improve the prognosis of the disease⁽⁴⁻⁷⁾. Cystoscopy and voided urine cytology (VUC) are the standard measures carried out to follow these patients. Cystoscopy carries the drawbacks of being invasive and costly⁽⁸⁻¹¹⁾ while VUC has low sensitivity to low and intermediate grade tumors, requires great

experience and still rather expensive⁽¹²⁾. In search for other diagnostic tools to replace, or at least reduce the frequency of cystoscopy, several urinary markers have been proposed and tested⁽¹³⁻¹⁴⁾. The marker we look fore should be accurate in diagnosis (in terms of sensitivity and specificity) and easy to perform (in terms of ease of the technique, availability and cost)⁽¹⁵⁾. Many of these urinary markers have provided better sensitivity than VUC but with reduced specificity⁽¹³⁻¹⁴⁾. To improve specificity, without compromising sensitivity, the idea of combining more than one urinary marker has evolved⁽¹⁶⁻¹⁸⁾. In this study, we evaluate three urinary markers: Survivine, Calreticulin (CRT) and urokinase Plasminogen Activator Receptor (uPAR) both

individually and in different combinations to detect recurrence in NMIBC.

2. Patients and Methods:

From March 2010 to October 2010, all patients with known history of NMIBC who are scheduled for follow-up cystoscopy in the Department of Urology, Alexandria University were included in this study prospectively. After getting the approval of the ethical committee in our institution, a well informed written consent is signed by the patient to collect a fresh voided morning urine sample and to obtain the necessary clinical and pathological data from his medical records. Approximately 50-100ml of morning voided urine sample was collected aseptically from every patient.

All patients underwent cystoscopy under general anaesthesia, and those who were found to have a definite or suspicious lesion(s) in the bladder underwent complete TURBT and the specimen was sent for histopathological assessment.

Voided urine samples were taken before cystoscopy, a portion of which was aliquoted into two ependorf tubes (1.5 ml each) and stored at -20°C till the time of the assay of urinary survivin and uPAR concentrations by ELISA technique, and the remaining portion was divided into 5ml aliquots in non adsorption modified tubes and then stored at -70°C until time of analysis of CRT by western blot technique.

All urine specimens were subjected to the following: measurement of survivin protein expression by enzyme-linked immunosorbent assay (ELISA)⁽¹⁹⁾, estimation of uPAR concentration samples by ELISA, and determination of CRT protein expression by western blot analysis⁽²⁰⁾. For western blotting, equivalent amounts of protein were separated by 10% SDS-PAGE and transferred onto nitrocellulose filter. The filters were first stained to confirm uniform transfer of all samples and then incubated in blocking solution for 2 hrs at room temperature. The filters were reacted with the anti-calreticulin antibody at a dilution of 1:1000 for 2 hrs. Then the blot was washed three times (5 minutes each) with 1x PBST (0.05% Tween in phosphate buffered saline PBS) and then washed for 10 min with Tris buffered saline (TBS) with shaking. Filters were then incubated with horseradish peroxidase-conjugated secondary antibodies of 1:1000 for 1 h. After the secondary incubation the membrane was washed 3 times (5min each) with TBST (0.05%Tween) and then washed again in TBS with shaking. 3,3'-Diaminobenzidine (DAB) substrate solution was prepared, then 30 μl hydrogen peroxide were added. After developing the color of the blot, the reaction was stopped after appearance of the expected

bands by pouring out the substrate and rinsing with distilled water repeatedly. As an internal control, Beta Actin antibody (Affinity-purified Sheep Anti-human/mouse/rat Actin Antibody from R&D systems) was used as control.

Statistical analysis:

Data were fed to the computer using the Predictive Analytics Software (PASW Statistics 18) Qualitative data were described using number and percent. Association between categorical variables was tested using Chi-square test. The distributions of quantitative variables were tested for normality using Kolmogorov-Smirnov test which revealed abnormal distribution of the data. Thus, non-parametric statistics were applied. Quantitative data were described using median, minimum and maximum as well as mean and standard deviation. Mann-Whitney was used to test compare between two samples while Kruskal-Wallis test was used to compare three or more samples. The diagnostic performance of the three marker to discriminate recurrence from no recurrence is evaluated using Receiver Operating Characteristic (ROC) curve analysis. Logistic regression technique was adopted to formulate equations for prediction of recurrence. The developed model was assessed using Model Chi-square and Nagelkerke's R². Significant test results are quoted as two-tailed probabilities. Significance of the obtained results was judged at the 5% level.

3. Results:

From March 2010 to October 2010, 68 patients were eligible to our study. Thirty patients were found to have no recurrence of the disease and were considered as group I (recurrence-free group) (18 males and 12 females with mean age of 44 ± 5 yrs) while 38 patients had non-muscle-invasive recurrence and were considered as group II (recurrence group) (22 males and 16 females with mean age of 47 ± 4 yrs). In the recurrence group, 24 patients had low grade recurrence (group IIa) while 14 patients had high grade recurrence (group IIb).

Individual Markers:

For the three tested markers, the urinary concentration of each marker individually was significantly higher in the recurrence group (group II) than in the recurrence-free group (group I). It was also higher in the high grade subgroup (group IIb) than in the low grade subgroup (group IIa) but the difference between subgroups was not statistically significant (Table 1).

The threshold values for optimal sensitivity and specificity of the investigated bladder cancer markers were determined by receiver operating characteristics

(ROC) curve. The cut off values that maximized the sum of sensitivity and specificity for discrimination between the two groups were chosen (Table 2).

As a single test, urinary survivin showed the highest sensitivity (84.37%), the highest specificity (100%) and the largest area under the curve (0.900) of the three markers (Fig. 1). Urinary uPAR and CRT had sufficiently high specificities (93.33% and 93.33% respectively) but their sensitivities were lower for clinical use (51-52%, 47.37% respectively). (Table 2).

Marker Combinations:

To study the usefulness of concomitant examination of urinary survivin, CRT and uPAR, we evaluated the diagnostic value of different marker combinations compared with that of a single use of survivin. Various combinations of the three markers were done and ROC curves were constructed. We defined the combined marker as positive when one or more of the individual markers were higher than their respective cut off levels. (OR combinations). (Table 3 & Fig. 2)

Combination of the three diagnostic markers survivin OR CRT OR uPAR gave the highest sensitivity 96.87% corresponding to a 12.50% increase than that of single use of survivin but at the cost of 20% lower specificity (80.00%) (Table 4)

Logistic Regression Model

It was clear that various combinations of the three urinary markers using Boolean operators improved the sensitivity for predicting bladder cancer, however that was at the cost of lower specificity. Therefore, logistic regression technique was adopted to formulate an equation using the three urinary markers in an attempt to improve both the sensitivity and specificity for prediction of bladder

cancer recurrence. The model's predictive power is significantly better than the model containing the constant only. (Model $X_3^2 = 40.112, p = .001$). The model accounts for 80.4% of the variability in cancer occurrence. Only CRT Western Blot and Survivin Elisa pg/ml are significantly contributing to the model. The equation resulting from this model is as follows:

$$\text{Model} = 1/(1+e^{-Y}), Y = -4.916 + [3.770 \times \text{CRT Western Blot (Urine)}] + [.186 \times \text{Survivin Elisa value pg/ml (Urine)}]$$

where CRT western blot (urine) equals zero if it is negative and equals one if it is positive.

The best cut off value for the logistic regression model was calculated by the ROC curve as >0.61 (score above 0.61 resulting from substitution of the equation will diagnose bladder cancer recurrence while score below or equal to 0.61 will exclude it). AUC was 0.954 (95% CI 0.850 to 0.993). At the best cut off value for the model the sensitivity was 93.75% and the specificity was 100.00%. The model score among the no recurrence group ranged from 0.02 to 0.61 (median: 0.60) while its range was from 0.09 to 1.00 (median: 1.00) and from 0.08 to 1.00 (median: 1.00) in the low grade and high grade recurrence subgroups respectively.

A direct comparison between the diagnostic performance of the new model, survivin alone and various combinations developed by Boolean operators showed that the new model had the highest sensitivity (93.75%) and the same specificity as survivin (100.00%). The statistical comparison between AUC of the new model and that of survivin alone showed a significant difference ($p = 0.025$). (Table 3)

Table 1: Urinary values of the three markers and difference between both groups and between low and high grade subgroups

Marker		G1 (n=30)	GII (n=38)		Test
			Low Grade GIIa N=24	High Grade GIIb N=14	
Survivin	Mean	15.53 ± 5.2	78.9 ± 49.77		u=141
	Median (range)	17.3 (6.3-23.5)	69.7 (12.1-183.6)		P=0.018*
	Median (range)		60.9 (12.1-136.1)	115.6 (13.6-183.6)	u=81 p=0.087
uPAR	Mean	1224.93 ± 619.46	2933.29 ± 2792.8		u=48
	Median (range)	956 (485-2459)	2005 (506.5-11175)		p<0.001*
	Median (range)		1624.5 (546-11175)	2136.25 (506.5-9254)	u=109 p=0.382
CRT	Pos/Neg	2/28	18/20		χ²=7075
	Positivity	6.7%	47.4%		p=0.005*
	Pos/Neg		10/14	8/6	χ²=0.849
	Positivity		41.7%	57%	p=0.357

Table 2: Diagnostic performance of individual markers.

Parameter	AUC (95% CI)	p-value	Cut-off value	Sen. (95% CI)	Sp. (95% CI)	PPV (95% CI)	NPV (95% CI)
Survivin	0.900	.0001	>23.5	84.37	100.00	100.0	75.0
uPAR	0.715	.0045	>19.62	51.52	93.33	94.4	46.7
CRT	0.704	.0064		47.37	93.33	94.7	41.2

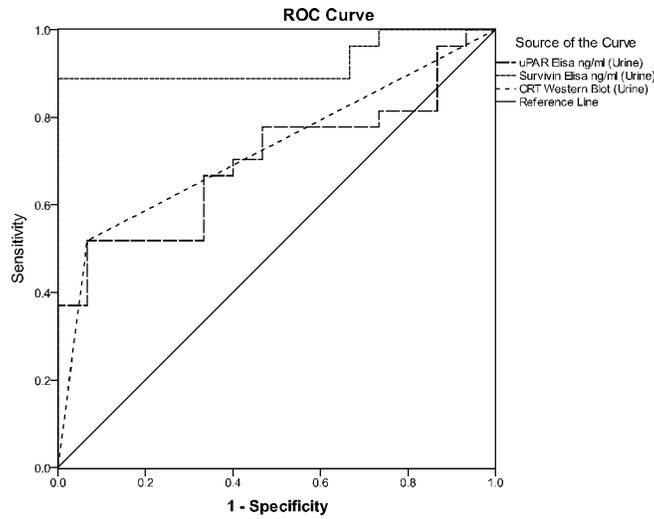


Figure (1): Comparison between diagnostic performance of the three urinary markers

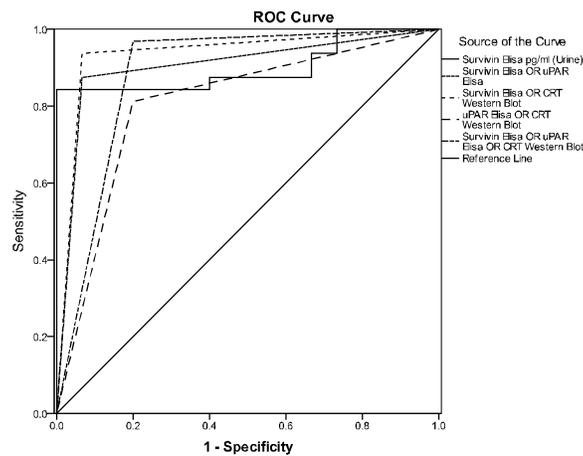


Figure (2): Comparison between diagnostic performance of survivin alone and various “OR” combinations of the three urinary markers.

Table (3): Comparison between diagnostic performance of the new model, survivin alone and various Boolean operator combinations.

Parameter	AUC (95% CI)	p-value	Sen. (95% CI)	Sp. (95% CI)	PPV (95% CI)	NPV (95% CI)	Diff. between areas (p-value)*
Model	0.954	.0001	93.75	100.00	100.0	88.2	
Survivin	0.900	.0001	84.37	100.00	100.0	75.0	0.054 (0.065)
Survivin OR uPAR	0.904	.0001	87.50	93.33	96.6	77.8	0.050 (0.064)
Survivin OR CRT	0.935	.0001	93.75	93.33	96.8	87.5	0.0188 (.619)
Survivin OR uPAR OR CRT	0.884	.0001	96.87	80.00	91.2	92.3	0.0698 (.162)
uPAR OR CRT	0.806	.0001	81.25	80.00	89.7	66.7	0.148 (0.017)

4. Discussion

Several clinical and molecular studies support the hypothesis that MIBC and NMIBC have different pathways. For the NMIBC, the pathway is characterized by certain molecular events that should be looked at when trying to detect recurrence^(17, 21-25)(15-20 in 9). In the present study, we initially evaluated the diagnostic potential of the three urinary proteins: survivin, CRT and uPAR in the detection of recurrence in patients with NMIBC.

Survivin, a member of the inhibitor of apoptosis protein (IAP) family, has a unique role in apoptosis, control of cell division and modulation of angiogenesis. It is also selectively expressed in malignant versus normal tissues. For that, it is expected to be an excellent diagnostic marker of bladder cancer⁽²⁶⁾. Using both protein and mRNA detection methods, Smith *et al.*, were the first to evaluate the diagnostic potential of urinary survivin in bladder cancer. Survivin protein and mRNA were detected in all 47 patients with new or recurrent bladder cancer. In contrast, only 3 of 35 patients with negative cystoscopy had detectable urinary survivin levels. They reported 100% sensitivity and 95% specificity. Shariat *et al.*,⁽¹⁹⁾ demonstrated that higher levels of survivin correlated with an increased risk of bladder cancer and higher grade tumors with a sensitivity of 64%. Similarly, Weikert *et al.*, detected survivin mRNA in urine of 68% (24 / 35) of patients by RT-PCR and considered it as a highly specific biomarker for TCC detection although it was not found to relate to pathologic stage or grade categories⁽²⁷⁾.

On the same track, Moussa *et al.*,⁽²⁸⁾ and Hou *et al.*,⁽²⁹⁾ reported the detection of survivin mRNA in cells isolated from urine sediments using RT-PCR and real time quantitative RT-PCR respectively. They stated that urinary survivin mRNA detection is directly related to tumor pathologic stage. Recently, Eissa and co-workers⁽³⁰⁾ reported a marked increase in the positivity rate of urine survivin mRNA in the malignant

group compared with the benign and healthy groups using qualitative RT-nested PCR with a sensitivity of 78.6%.

Compared with the aforementioned studies, the sensitivity of urinary survivin for bladder cancer detection reported by our study (84.37%) was lower than initially reported by Smith *et al.* (100%)⁽³¹⁾ but similar to that of Eissa *et al.*, (78.6%)⁽³⁰⁾. The discrepancy in results may be attributed to different sample sizes and types, as some of the current study cases were associated with schistosomiasis.

In a search for candidate proteins as new bladder tumor markers, proteome differential display using two dimensional electrophoresis (2DE) was used and led to the identification of urinary CRT.⁽³²⁾ Calreticulin (CRT), a unique endoplasmic reticulum (ER) luminal Ca²⁺ - binding chaperone, is a multifunctional molecule related with glycoprotein maturation and chaperone function, Ca²⁺ homeostasis, cell adhesion, cell signaling, regulation of gene expression, nuclear transporting mechanisms and autoimmunity⁽³³⁾. Although an increase of CRT expression in tumor cells and proliferating cells is well known, details of the mechanism of this increase are as yet undetermined. It has been identified in hepatocellular carcinoma⁽³⁴⁾ and poorly differentiated colon cancer⁽³⁵⁾. In bladder cancer, Celis⁽³⁶⁾ showed that some molecular chaperones, including CRT, were increased in primary cultures derived from low grade superficial bladder TCC. Clinically, Kageyama *et al.*,⁽³²⁾ demonstrated increased CRT expression in bladder cancer tissue using proteomic analysis and quantitative WB analysis. They also reported a sensitivity of 73% at a specificity of 86% for urinary CRT in detecting bladder cancer patients. Recently, the same group⁽³⁷⁾ further evaluated the potential suitability of CRT as urinary marker for bladder cancer using ELISA and reported a lower sensitivity of 67.9% at specificity of 80%.

In our work, CRT also correlated with bladder cancer recurrence with a sensitivity of 47.37% and

specificity of 93.33%. Its expression was significantly higher in the group of patients who developed recurrence.

During several steps of tumor growth and progression, proteolytic degradation of the ECM is required. Among the proteases that play an active role in the degradation of the ECM are the plasminogen activation system⁽³⁸⁾. Among the uPA system components, uPAR might have a more crucial role in tumour progression, since many of the activities of uPA, including its activation by plasmin, are dependent on its binding to uPAR⁽³⁹⁾. Over expression of uPAR antigen in bladder cancer has been reported by several investigators and was found to correlate with tumor stage, grade⁽⁴⁰⁻⁴¹⁾, invasiveness⁽⁴²⁾, outcome⁽⁴³⁾ and survival⁽⁴⁰⁾.

The present study revealed the significant increase of urinary levels of uPAR in the malignant group compared to the control group. Our results do not differ from other studies. Shariat *et al.*,⁽⁴⁴⁾ found that elevated uPAR urinary levels were significantly higher in bladder cancer patients than in healthy individuals. Similarly, Casella *et al.*,⁽⁴⁵⁾ tested uPAR and uPA before cystoscopy and showed that uPAR could help to find high risk patients for bladder cancer. In addition, EcKe and co workers⁽⁴⁶⁾ reported that pathological concentrations of uPAR are detectable in the serum and in urine of bladder cancer patients.

Neither the source of soluble uPAR in human body fluids nor the mechanism of receptor release from the cell surface has been clearly determined⁽⁴⁷⁾.

But, Understanding the molecular biology of bladder cancer, it is unlikely that a single molecular marker can detect all bladder cancers⁽¹⁶⁾. In an attempt to improve the sensitivity and the specificity for diagnosis of bladder cancer, different combinations of the three studied markers were tried to achieve the highest sensitivity and specificity.

To our knowledge, the possible utility of concomitant use of urinary levels of survivin, CRT and uPAR as diagnostic tool in bladder cancer has not been evaluated previously. However, others have compared the results of multiple markers including CRT and survivin. Iwaki *et al.*,⁽⁴⁸⁾ compared the sensitivity and specificity of CRT, α -synuclein and catechol-o-methyltransferase when used alone or in combinations. They found out that the sensitivity and specificity of the combined marker were 76.8% and 77.4% respectively corresponding to a 5.4% higher sensitivity and a 0.4% lower specificity compared with a single use of CRT. In addition, Eissa *et al.*,⁽³⁰⁾ in a direct comparison between urine cytology, survivin, and tissue inhibitor matrix metalloproteinase-2 (TIMP-2) showed that TIMP-2 had the highest sensitivity (93%), whereas urine cytology exhibited the highest specificity (100%). Combination of urine cytology with TIMP-2

gave same sensitivity (93%) and specificity (83.7%) as those obtained with TIMP-2 alone. Combined use of the three urine markers improved the sensitivity up to 98% but at the expense of specificity (76.7%). The combined use of only survivin and TIMP-2 as promising urinary markers is recommended where the sensitivity remains 98% but the specificity was raised to reach 79%.

In this study, a direct comparison between the three studied markers showed that survivin had the highest sensitivity 84.37% and exhibited the highest specificity (100%). So we evaluated the diagnostic value of the combined marker compared with that of a single use of survivin. Combined use of the three urinary markers gave the highest sensitivity (96.87%) but at the expense of lower specificity (80%). The combined use of only survivin and uPAR improved the sensitivity to 87.50% at the expense of 6.67% lower specificity. The sensitivity and specificity of the combined marker survivin and CRT were 93.75% and 93.33% respectively, corresponding to 9.38% higher sensitivity and a 6.67% lower specificity compared with a single use of survivin.

Although various combinations of the three urinary markers using Boolean operators improved the sensitivity for predicting bladder cancer than single use of survivin however that was at the cost of lower specificity. Therefore logistic regression technique was adopted in a trial to formulate an equation using the three urinary markers in an attempt to improve both the sensitivity and specificity for prediction of bladder cancer. Only CRT western blot and survivin Elisa were contributing to the model.

A direct comparison between the diagnostic performance of the new model, survivin alone and various combinations developed by Boolean operators showed that the new model had the highest sensitivity (93.75%) and the same specificity as survivin (100.00%). However, the statistical comparison between AUC of the new model and that of survivin alone doesn't reach the level of statistical significance ($p = 0.065$).

Therefore, the use of the logistic regression developed model as a promising urinary marker for early detection of bladder cancer recurrence is recommended where the specificity remains as high as that of survivin (100.0%) but the sensitivity was raised to 93.75%.

Conclusion:

Combining more than one urinary marker is a logic step forward that improves the sensitivity of detection of bladder cancer recurrence. The use of this logistic regression model as a promising urinary marker for early detection of bladder cancer recurrence is recommended where the specificity remains 100.0%

while the sensitivity is raised to 93.75%. However larger studies should be carried out to prove the usefulness of these marker combinations.

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