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Evaluation of tissue and urinary survivin expression in non-muscle-invasive bladder cancer

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Abstract
Introduction: Approximately 70% of bladder cancers are non-muscle-invasive (NMIBC), and respond well to endoscopic transurethral resection. However, 70% of these patients experience tumor recurrence. As the tendency for local recurrence and/or progression extends over the lifetime, patients with superficial bladder cancer must undergo life-long surveillance. Combination of cystoscopy and urine cytology is considered the "gold standard" for this surveillance. However, they suffer from drawbacks where cystoscopy is an invasive procedure and urine cytology shows limited ability to detect low grade bladder tumors. Therefore, new non-invasive tests with high sensitivity and specificity that are easy to perform are needed not only for initial diagnosis but also in surveillance for recurrent tumors.
Objective: To investigate the magnitude of survivin expression in non-muscle-invasive bladder cancer and its possible value as a non invasive diagnostic tool.
Patients and methods: From March 2010 to October 2010, 68 patients with known history of NMIBC who were scheduled for follow-up cystoscopy in the department of Urology, Alexandria University were included in this study prospectively. 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. Survivin expression was determined in urine and in bladder cancer tissue both by Western blotting and by ELISA.
Results: The study included 68 patients. Tumor recurrence was detected in 38 patients, of whom, 24 had low grade recurrence. The urinary concentration of survivin was significantly higher in the recurrence group

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by both detection methods ($U = 141$, $P = 0.018$ and $\chi^2 = 10.46$, $P = 0.001$ for ELISA and WB respectively). Survivin by ELISA showed higher sensitivity and specificity (84.4% and 100%) than that by WB (55.3% and 93.3%). In tumor tissue, by both methods, survivin was detected in higher levels than in urine but there was no significant correlation between urinary and tissue levels neither in the whole recurrence group nor in the low grade subgroup.

Conclusion: Urinary survivin is a useful marker for non-invasive detection of non-muscle-invasive bladder cancer recurrence. Its detection is better using ELISA technique than WB and there is no correlation between its expression in tissue and urine.

Introduction

Approximately 70% of bladder cancers are non-muscle-invasive (NMIBC), and respond well to endoscopic transurethral resection. However, 70% of these patients experience tumor recurrence [1,2]. As the tendency for local recurrence and/or progression extends over the lifetime, patients with superficial bladder cancer must undergo life-long surveillance [3,4]. Combination of cystoscopy and urine cytology is considered the "gold standard" for this surveillance. However they suffer from drawbacks where cystoscopy is an invasive procedure [5] and urine cytology shows limited ability to detect low grade bladder tumors [6]. Therefore, new non-invasive tests with high sensitivity and specificity that are easy to perform are needed not only for initial diagnosis but also in surveillance for recurrent tumors [7,8].

Survivin is a bifunctional protein that regulates cell division and suppresses apoptosis. It is the smallest member of inhibitor of apoptosis (IAP) family of proteins. Although it is abundantly expressed in fetal tissues [9], it is undetectable in most normal, terminally differentiated adult tissues. However, survivin is over-expressed in a variety of human cancers, suggesting that reactivation of the survivin gene frequently occurs in cancers [10]. The cancer-specific expression of survivin, coupled with its importance in inhibiting cell death, and in regulating cell division, makes it a useful diagnostic marker of cancer and a potential target for cancer treatment [11].

The aim of this work was to investigate the magnitude of survivin expression in non-muscle-invasive bladder cancer and its possible value as a non invasive diagnostic tool.

Patients and methods

From march 2010 to October 2010; all patients with known history of NMIBC who were 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 was signed by the patient to collect a fresh voided morning urine sample, a piece of the resected tumor and to obtain the necessary clinical and pathological data from his medical records. Approximately 50–100 ml 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 epindorf tubes (1.5 ml each) and stored at −20 °C till the time of the assay of urinary survivin concentrations by ELISA technique [12], and the remaining portion was divided into 5 ml aliquots in non adsorption modified tubes and then stored at −70 °C until time of analysis of survivin by Western blot technique [13].

Tumor tissue specimens were obtained from patients by transurethral resection (TUR) and were histologically verified. Staging and grading according to the union international contre le cancer (UICC) [14] and 2004 WHO criteria [15] was carried out by an experienced pathologist. Fresh tissues were stored immediately at −70 °C until preparation of the sample used for survivin protein quantification by both ELISA and Western blot analysis.

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 compare between two samples. Correlations between two quantitative variables were assessed using Spearman's rho test. The diagnostic performance of survivin expression was evaluated using Receiver Operating Characteristic (ROC) curve analysis. Kappa statistic was performed to determine consistency between survivin in urine and in tissue. Significance of the obtained results was judged at the 5% level. It is quoted as two-tailed probabilities.

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) while 38 patients had non-muscle-invasive recurrence and were considered as group II (recurrence group). Demographic data of both groups are presented in Table 1.

Table 1 Demographic data of all patients.

Group	G1 Recurrence-free	G2 Recurrence
No.	30	38
Age	34–72 44 ± 5	39–76 49 ± 8
Sex		
Male	18	22
Female	12	16
Grade		
Low		24
High		14

Urinary survivin

We evaluated the potential diagnostic value of survivin detection in urine using two methods: ELISA and WB analysis

By ELISA

Urinary survivin concentration of the recurrence-free group ranged from 6.30 to 23.50 pg/ml (median: 17.30) with a mean value of 15.53 ± 5.20 pg/ml, while its range was from 12.10 to 183.60 pg/ml (median: 69.70) in the recurrence group with a mean value of 78.90 ± 49.77. Statistical comparison between the median values of survivin in the two studied groups using the non parametric Mann Whitney *U* test showed a significant difference ($U = 141.00$, $P = 0.018$) indicating a significant association between its level and bladder cancer recurrence (Table 2).

Although, the median values were approximately two times higher in group Ib (high grade bladder tumor) than group Ia (low grade bladder tumor), a non significant difference was found between the two groups ($U = 81.00$, $P = 0.087$) (Table 2).

At the best cut off value for survivin, the sensitivity was 84.37% and the specificity was 100% (Fig. 1 and Table 3). Among the low grade subgroup, survivin showed a true-positive rate of 58.3% (14 out of 24 patients were above the cut off value 23.5 pg/ml) while in the high grade subgroup 13 out of 14 were above the cut off value (positivity rate of 92.9%).

By Western blot

The positivity rate for survivin in the voided urine samples of the recurrence-free group was 2/30 (6.7%), while the positivity rate in the recurrence group was 21/38 (55.3%). Statistical comparison between the positivity rates of urinary survivin in the two studied groups using non parametric chi-square test showed a significant difference ($\chi^2 = 10.46$, $P = 0.001$) indicating a significant association between its expression and bladder cancer recurrence. However, a non significant difference was found between group IIa (low grade) and group IIb (high grade) ($\chi^2 = 2.34$, $P = 0.126$) (Table 2).

The overall sensitivity and specificity of urinary surviving detection by WB for predicting bladder cancer recurrence were 55.26% and 93.33% respectively (Table 3).

Our findings revealed that urinary survivin levels detected by ELISA and WB were significantly associated with bladder cancer. However, in a direct comparison of both survivin protein detection assays, the survivin ELISA showed a higher sensitivity and specificity than survivin WB but the difference does not reach statistical significance (differences between areas = 0.121, $P = 0.080$) (Table 3 and Fig. 1).

Tissue survivin

In our study, we investigated the expression of survivin in bladder tumor tissue samples by both ELISA and WB. The tumor tissue level of survivin by ELISA ranged from 15.00 to 2108.00 pg/ml (median = 437.75) with a mean value of 1022.42 ± 1061.74 pg/ml. On the other hand, survivin protein was detected by WB in tumor tissue of 27 patients with tumor recurrence with a positivity rate of 71.1%.

Although the median levels of survivin were approximately 6 times higher in tumor tissue than in urine, no significant correlation was observed between urine and tissue levels neither in the whole malignant group nor in the low grade subgroup. However, a significant positive correlation was found between tissue and urinary levels of survivin in the high grade group ($r = 0.645$, $P = 0.013$) (Table 4 and Fig. 2). Similar correlations were reported for survivin WB (Table 5).

Discussion

Deregulation of apoptosis is a hallmark in human carcinogenesis, and bladder cancer has been shown to resist programmed cell death with altered expression of pro and anti-apoptotic proteins [16]. Survivin, a member of the inhibitor of apoptosis protein (IAP) family, has a very important role in apoptosis and control of cell division. At the same time it is selectively expressed in malignant versus normal tissues. These two characteristics make survivin an excellent diagnostic biomarker in bladder cancer [17].

Table 2 Survivin expression in the two groups by both techniques.

		Group 1 $N = 30$	Group 2 $N = 38$		Test
			G2a $N = 24$	G2b $N = 14$	
Survivin By ELIZA	Mean	15.53 ± 5.2	78.9 ± 49.77		$U = 141$
	Median	69.7 (12.1–1836.6)	17.3 (6.3–23.5)		$P = 0.018^*$
	Mean		60.9	115.6	$U = 81$
	Median		12.1–136.1	13.6–183.6	$P = 0.087$
Survivin By WB	+ve/−ve	2/28	21/17		$\chi^2 = 10.46$
	Posetivity	6.7%	55.3%		$P = 0.001^*$
	+ve/−ve		11/13	10/14	$\chi^2 = 2.34$
	Posetivity		45.8%	71.4%	$P = 0.126$

* Significant at $P < 0.05$.

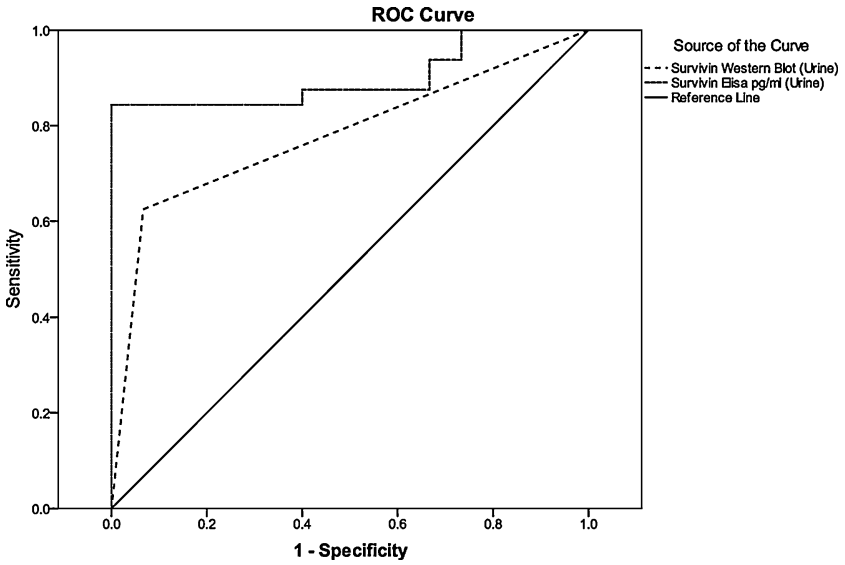

Figure 1 Comparison between the diagnostic performance of both survivin ELISA and WB: AUC equals 0.900 and 0.743 respectively.

Table 3 Comparison between the diagnostic performance of survivin ELISA and WB determined by ROC analysis.

Parameter	AUC (95% CI)	*P*-value	Cut-off value	Sen. (95% CI)	Sp. (95% CI)	PPV (95% CI)	NPV (95% CI)	Diff. between areas (*P*-value)
Survivin ELISA pg/ml (urine)	0.900 0.777–0.968	0.0001	>23.5	84.37 67.2–94.7	100.00 78.0–100.0	100.0	75.0	0.121 (.080)
Survivin WB (urine)	0.743 0.604–0.853	0.0005		55.26 38.3–71.4	93.33 68.0–98.9	95.5	45.2	

Table 4 Correlations between tissue and urinary levels of survivin.

	S in T by ELISA	S in U by ELISA	
Group 2 $N=38$	437.75 (15.00–3404.00)	69.7 (12.00–183.00)	$r_s=0.243$ $P=0.142$
Group 2a $N=24$	322.50 (15.00–3404.00)	60.90 (12.10–136.10)	$r_s=0.022$ $P=0.920$
Group 2b $N=14$	881.40 (93.40–2908.00)	115.60 (13.60–183.60)	$r_s=0.645$ $P=0.013$

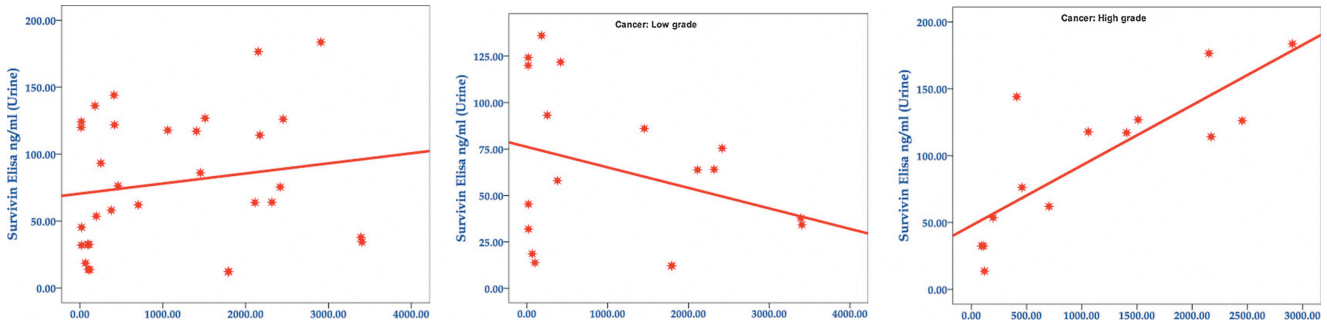

Figure 2 Correlations between tissue and urinary levels of survivin.

Table 5 Agreement between tissue and urinary expression of survivin WB in group I, group Ia and group Ib patients.

	Agreement No. (%)	No agreement No. (%)	Kappa (*P*-value)
Group I	24 (63.16)	14 (36.84)	0.299 (0.135)
Group Ia Low grade	12 (50.00)	12 (50.00)	0.020 (0.916)
Group Ib High grade	12 (85.71)	2 (14.29)	0.588* (0.016)

* Significant at $P < 0.05$.

In the current study, we investigated survivin protein expression in both tumor tissue and urine samples using ELISA, confirmed by Western blotting WB. A strong expression of survivin protein was detected by ELISA in all 38 tumor tissue samples. Additionally, 71% (27/38) of the investigated tissue samples showed detectable amounts of survivin by WB. Taken together, our data revealed strong survivin protein expression in bladder tumor tissues indicating that this protein might play an important role in carcinogenesis of the human urinary bladder.

The results of this study were in agreement with those of Wu et al. [18], who reported high survivin protein expression detected by Western blot analysis in 76.6% of bladder tumor tissue extracts and no detectable levels in normal tissues. Similarly, Xiao et al. [19], reported the expression of survivin protein in tumor tissues derived from patients with TCC of the urinary bladder and that it was significantly associated with tumor grade. In line with the previous finding, Swana et al. [20] and Ku et al. [21], revealed a high incidence of survivin protein expression in bladder cancer tissue using immunohistochemical-staining. In addition, Schultz et al. [22] reported an elevated survivin mRNA expression in urothelial cell carcinomas determined by RT-PCR assays.

Moreover, no significant association between survivin expression and tumor grade was found in the present study, which disagrees with Swana et al. [20], who reported that survivin detection correlated closely to high tumor grade. However, consistent with our results, Ku et al. [21], failed to detect a correlation between survivin expression and tumor grade in 88 non-muscle invasive bladder tumors. The discrepancy in results may reflect differences in the methods used to detect survivin.

Based on the fact that many tumor associated or derived molecules are potentially released into the urine when it comes in contact with the tumor, many non invasive urine based immunoassays have been designed to measure these molecules for detecting bladder cancer [23]. As survivin is selectively expressed in malignant epithelium and in the same time present in detectable levels in urine, it is considered as an attractive urinary biomarker for detection of bladder cancer [17].

In the early work by Smith et al. [24], urinary survivin protein and mRNA had a sensitivity of 100% and specificity of 95% in the detection of new or recurrent cases of bladder cancer. In a large study by Shariat et al. [12], higher levels of survivin were found to correlate with an increased risk of bladder cancer and higher grade tumors. In this study survivin sensitivity was 64%.

Consistent with these previous reports, the present study findings revealed a significant increase of survivin protein content in urine of the malignant group compared to the tumor-free group on using both ELISA and WB assays. However, it did not significantly differ with respect to the low and high grades of bladder tumor.

From the ROC curve, survivin ELISA sensitivity was found to be 84.57% whereas specificity was 100%. Upon using the WB detection assay lower sensitivity (55.3%) and lower specificity (93.33%) were reported. From the data presented here, the ELISA assay seems to be more sensitive and more specific in detecting urine survivin than the WB assay, though statistical comparison between the two methods using differences between AUC test showed a non significant difference ($P = 0.08$). Similarly, in a direct comparison of both survivin protein detection assays, Kappler et al. [13], reported a higher sensitivity and a stronger correlation to prognosis of survivin ELISA in detecting soft-tissue sarcoma (STS) patients compared with WB assay. This could be explained with the different detection limits. Another reason for differences between the two protein detection assays could be the use of different antibodies, which recognize different antigen epitopes and may differ in their sensitivities [13].

Though urinary survivin levels did not correlate significantly with tumor grade, the findings obtained from the current study have provided further evidence that survivin detection is a highly specific marker for bladder cancer. This is in accordance with Weikert et al. [25], who were able to detect survivin mRNA in urine of 68% (24/35) of patients by RT-PCR, and announced urinary survivin as a highly specific biomarker for TCC detection, though it did not relate to pathologic stage or grade categories.

In line with the previous finding, Moussa et al. [26], and Hou et al. [27], reported the detection of survivin mRNA in cells isolated from urine sediments using RT-PCR and real time quantitative RT-PCR respectively. However, they stated that urinary survivin mRNA increased progressively in accordance with the depth of TCC infiltration in the muscles. Recently, Eissa and co-workers [28], 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. They reported a sensitivity of 78.6%.

Compared with the aforementioned studies, the sensitivity of urinary survivin for bladder cancer detection reported by our study was lower than initially reported by Smith et al. [24] (100%) but in good accordance with the recent study of Eissa et al. [28]. Upon using a biodot microfiltration detection system to detect survivin in voided urine specimens, lower sensitivity (64%) and lower specificity (93%) were reported by Shariat and colleagues [12]. The discrepancy in results may be attributed to different sample sizes and types, as some of the current study cases were associated with schistosomiasis.

Regardless of whether survivin detection strategies in urine samples are based on protein or mRNA analysis, they should yield comparable results since survivin is a short-lived non secreted protein and detection is dependent on its abundance in exfoliated malignant cells [29]. Therefore, our results, consistent with the studies discussed above provide support to incorporating survivin expression in urine as one of potential markers being developed for bladder cancer detection.

Conclusion

Urinary survivin can be considered as a promising non-invasive marker for early detection of recurrence of NMIBC. Although, the results of ELISA and WB detection methods were comparable, the strongest diagnostic statement can be made using the more sensitive ELISA. Its expression in urine does not correlate with that in tumor tissue.

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