Survivin Expression as a Strong Indicator of Recurrence in Urothelial Bladder Cancer. Predictive Value of Nuclear *versus* Cytoplasmic Staining

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Abstract. *Background: Since its discovery in 1997, survivin has garnered significant interest due to its putative role as an inhibitor of apoptosis. Few studies have investigated the immunohistochemical status of survivin in urothelial bladder carcinoma. The subcellular localization of survivin (nuclear, cytoplasmic) and its differential predictive value is a parameter that previous studies have almost ignored. The aim of this study was to investigate the expression pattern of survivin in order to determine its potential prognostic significance. Materials and Methods: Archival tumor tissue from 80 patients with urothelial carcinoma were analysed by immunohistochemistry. Results: Nuclear and cytoplasmic positive scores of 61.25% (49/80) and 22.5% (18/80), respectively were found. Nuclear positive staining correlated strongly with increased grade (p=0.001), stage (p=0.039) and the probability of tumor recurrence (p=0.029). No relationship was found between the cytoplasmic survivin level and the clinicopathological parameters. Nuclear expression was identified as a significant independent predictor of relapse-free survival (p=0.016). Conclusion: Nuclear expression of survivin reflects an adverse disease outcome.*

Urothelial bladder cancer is the second most common genitourinary tumor and constitutes an important cause of morbidity and mortality (1). It presents a meaningful heterogeneity in biological behaviour that is not completely understood. It is well known that two morphologically similar tumors presenting in any assigned stage may behave in different fashions, a fact that seriously impedes the potential to accurately predict the clinical outcome in a given case. The ability to designate those superficial tumors with an adverse disease outcome and those unlikely to become invasive or clinically threatening would be of great clinical benefit. Superficial tumors that maintain a more malignant phenotype may be better treated with early aggressive therapy.

Survivin is a recently characterized novel member of the inhibitor of apoptosis (IAP) family. In addition to its anti-apoptotic function, survivin has been demonstrated to regulate cell division through the proper assembly of the bipolar mitotic spindle and segregation of chromosomes (2). Considerable evidence exists implicating survivin in angiogenesis. It is expressed during embryonic and fetal development, selectively overexpressed in common human carcinomas and completely down-regulated in normal adult tissue. A significant positive colleration between survivin levels and microvessel density has been discovered in various solid tumors (3, 4). Based on the detection of protein by immunohistochemistry and mRNA by polymerase chain reaction techniques, the overexpression of survivin has been reported in various human malignancies. Retrospective analyses of several solid tumors have linked survivin expression to decreased overall survival, aggressive disease, resistance to chemotherapy and accelerated rates of recurrence. A higher survivin expression has been correlated with an unfavorable survival or disease recurrence in colorectal cancer, particularly in stage II disease (5), in esophageal cancer (6), hepatocellular carcinoma (7), lung cancer (8), ovarian cancer (9), glioma (10), leukemias (11), neuroblastoma (12), melanoma (13) and non-melanoma skin carcinomas (14). Survivin expression has been shown in bladder cancer at varying rates and conflicting data in the literature exist regarding its association with outcome

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parameters. The aim of the present study was to investigate the possible correlation and the impact of this pivotal cancer protein on the prognosis of urothelial bladder carcinoma. The differential predictive value of nuclear *versus* cytoplasmic staining of survivin was investigated.

Materials and Methods

Study population. The study group comprised sequential eighty specimens (n=80) of primary urothelial carcinoma of the urinary bladder obtained by transurethral resection or total cystectomy. All the patients had been treated and followed at the same institution between 1998 and 2005. The cohort of patients in this study was highly selected, fairly homogenous and treated in a prospective and standardized fashion. The samples were processed anonymously. The demographic information, clinical presentation, pathological stage and follow-up were extracted from the medical charts. Tumor grading and staging were determined according to the principles outlined by the World Health Organization (WHO 2004) (15) and the TNM classification of the International Union Against Cancer (UICC) (16). The study population consisted of 69 men and 11 women with a mean age of 65 years (range 26-85) and a mean follow-up time after initial diagnosis of 33.9 months (range 12-96). A relatively large number of the patients had early-stage disease (PTa: 51 patients, PT1: 15 patients, ≥PT2: 14 patients).

Immunohistochemistry. One paraffin block was selected for each case. The major criterion of selection was good preservation of morphology. Cauterized or quantitatively inadequate material was avoided. Only sections containing sufficient epithelium to assess the antibody reactivity in 1,000 cells were considered eligible for this study. A mouse monoclonal antibody (clone 8E2, Abcam, Cambridge, Massachusetts, USA) against survivin protein at a 1:200 dilution was used. The immunohistochemistry protocol for this antigen was carried out on 4 μm-thick paraffin sections of the corresponding blocks. The marker was applied to the sections using a Bond-X, automated staining system (Vision Biosystems, Newcastle upon Tyne, UK). This specific assay is based on a soluble, dextran-polymer system which yields a high signal to noise ratio and minimizes any background that may be caused by endogenous biotin. Polymer-based detection technology is an advancement in immunohistochemical visualization chemistry. The sections, after peroxidase blocking, were incubated with the primary antibody for 60 min at room temperature and then incubated with horseradish peroxidase labeled polymer (HRP LP) for 30 min. The antigen–antibody reaction was visualized using 3-3'diaminobenzidine tetrahydrochloride (DAB) as the chromogen substrate. Finally, the tissue sections were slightly counterstained with hematoxylin for 30 s, dehydrated and mounted. The omission of the primary antibody in simultaneously incubated sections was used as the negative control. Three hundred cells were examined and selected among at least five non-consecutive fields and chosen in the most viable areas of the lesions at ×400 magnification in order to quantify the survivin expression. On the basis of percentage of survivin-positive cells, the lesions were classified as negative (0-10%) and positive (>10%), as proposed previously (17). All the immunohistochemical slides were analyzed by two independent pathologists. The interobserver variability was low. In cases of disagreement, a final score was determined by consensus after re-examination. Assessment of all the staining results was blinded to knowledge of the clinical outcome of the patients.

Table I. *Correlation between clinicopathological parameters and survivin staining patterns.*

Characteristic		Survivin subcellular expression					
		Nuclear expression			Cytoplasmic expression		
		+	–	*p*-Value	+	–	*p*-Value
Gender							
Male	69	44	25	0.322	16	53	0.712
Female	11	5	6		2	9	
Age							
<65 years	30	16	14	0.260	5	25	0.333
≥65 years	50	33	17		13	37	
Grade							
Low	52	25	27	**0.001**	15	37	0.064
High	28	24	4		3	25	
Stage							
Superficial	66	37	29	**0.039**	17	49	0.436
Muscle-invasive	14	12	2		1	13	

Statistical analysis. The correlation of survivin expression with clinical parameters was calculated using the Chi-square test. Disease-free survival and overall survival rates were estimated using the Kaplan-Meier algorithm for incomplete observations. Cumulative survival curves were compared using the log-rank test. The overall survival time was defined as the interval between the date of diagnosis and the last date when the patient was known to be alive (censored) or date of death for any reason (uncensored). The disease-free survival rate was measured as the period of time between the date of diagnosis and the date of the last follow-up examination in which the patient was disease free (censored), or the date of first recurrence whether it was a local, regional or distant recurrence (uncensored). A Cox proportional hazards ratio model was used to determine the independent predictors of relapse-free and overall survival using factors significant on univariate analysis as covariates. In all cases, a p-value ≤0.05 was considered to be statistically significant. Statistical analysis was performed using the SSPS software program 16.0 (SSPS Inc, Chicago, IL, USA).

Results

The associations between survivin expression and clinicopathologic factors are shown in Table I. A summary of the results of the Chi-square analysis of the studied variables is also displayed in the same table. According to the chosen cut-off point at 10%, nuclear and cytoplasmic positive scores of 61.25% (49/80) and 22.5% (18/80), respectively were found. Expression patterns of survivin staining are depicted in Figure 1. There was an obvious predominant nuclear staining pattern in 49 cases and a diffuse faint cytoplasmic staining in 18 samples. Coexistence of nuclear and cytoplasmic staining was observed in 13 cases. During the follow-up time, disease

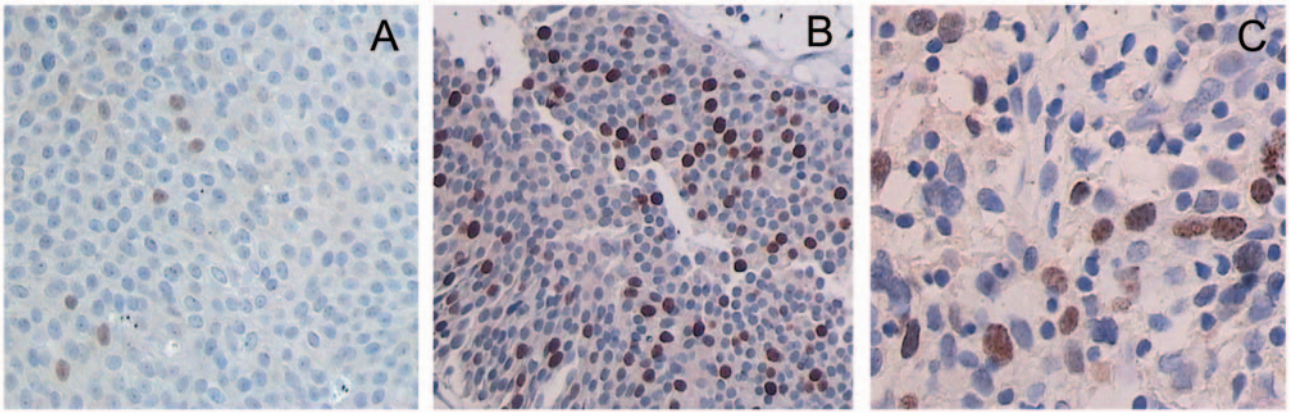

Figure 1. *Survivin expression patterns. A, Negative expression in a low-grade tumor. Positive staining limited to sporadic nuclei (×200). B, Positive staining in 25% of tumor cells. This low-grade tumor recurred 14 months after initial resection (×200). C, Positive staining in a high-grade tumor. About 20% of tumor cells demonstrate an obvious strong positive nuclear staining (×400).*

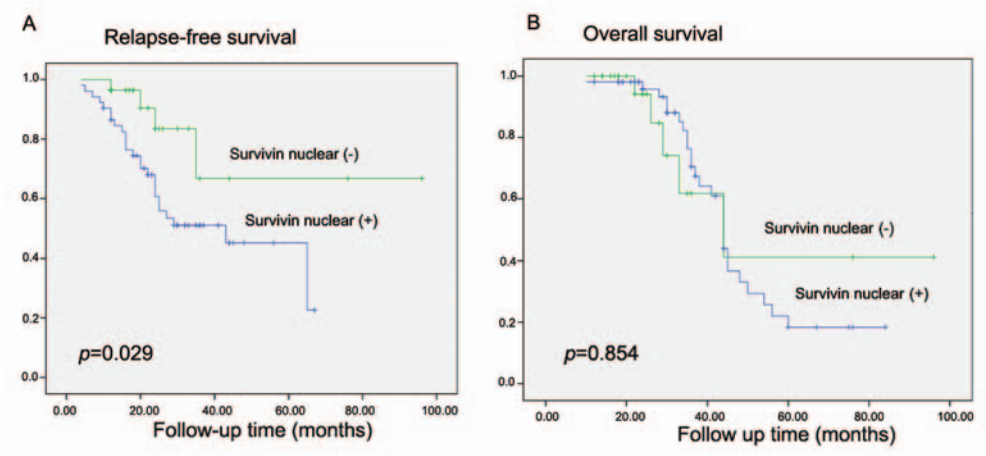

Figure 2. *Univariate survival analysis by nuclear expression of survivin. Kaplan-Meier curves of relapse-free (A) and overall survival (B).*

recurred in 29 out of the 66 patients with superficial tumor (44%) and progressed in 15 patients (23%). At the time of writing, 31 patients had died. No relationship between cytoplasmic survivin level and the clinicopathological parameters was demonstrated. In contrast, nuclear positive staining correlated strongly with increased grade (p=0.001) and stage (p=0.039), as determined by the Chi-square test. Kaplan-Meier analyses showed that positive nuclear expression of survivin was associated with an increased probability of tumor recurrence (log rank test, p=0.029) (Figure 2A). The survivin scores did not correlate with overall survival probability analyzing all the patients (log rank test, p=0.854) (Figure 2B). Using the Cox proportional hazards methods, a multivariate analysis was performed to assess the independent predictive value of all the significant markers for the overall and disease-free survival (Table II). The multivariate analysis revealed that the nuclear survivin expression was a strong independent predictor of disease recurrence (p=0.016) (Table II).

Discussion

By immunohistochemical analysis survivin is localized in two subcellular areas (cytoplasmic and nuclear). Li *et al*. (18) reported a satisfied exegesis based on its functional role. The nuclear distribution of survivin is associated with its role in promoting cell proliferation whereas the cytoplasmic pool of survivin may participate in controlling cell survival. A second explanation suggests that different splice variants of survivin may differ in their subcellular localization. Alternative splicing of the human survivin gene can give rise

Table II. *Cox proportional hazard model analysis*.

	Relapse-free survival			Overall survival		
	Hazard ratio	*p*-Value	95% CI	Hazard ratio	*p*-Value	95% CI
Grade	1.283	0.564	0.550-2.990	2.596	0.055	0.979-6.880
Stage	0.000	0.961	0.000-3.189	1.798	0.210	0.719-4.501
Nuclear survivin	0.265	0.016	0.900-0.782	1.287	0.629	0.463-3.577

Grade: low *versus* high; Stage: superficial (PTa, PT1) *versus* muscle invasive tumors (≥PT2); Nuclear survivin: positive (>10%) *versus* negative (0-10%); CI: confidence interval.

to five different mRNA isoforms (19-21) that can be found in different subcellular compartments. The anti-survivin antibodies recognize them all due to the existence of an identical amino terminal peptide in all survivin variants. A third opinion reported by Chen *et al*. (22) devalued the cytoplasmic staining of survivin in urothelial bladder cancer suggesting that the polyclonal antibodies used in previous studies tended to generate nonspecific background staining and they used a monoclonal antibody in their study to avoid this potential pitfall. The survivin immunohistochemistry pattern thus observed was predominantly nuclear, with focal weak cytoplasmic stain in some cases that were not found to correlate with tumor grade. In the present study, using a monoclonal antibody for survivin in combination with the sensitive Bond polymer system, a clear immunohistochemical pattern was achieved similar to that of Chen *et al*. In the present research, in 49 cases there was an obvious nuclear staining pattern and 18 specimens revealed diffuse faint cytoplasmic staining. Although the cytoplasmic staining was very weak resembling non-specific staining, the cytoplasmic expression was scored, but no statistical correlation with the variables was found.

Original reports of cytoplasmic or nuclear survivin localization in various neoplasms have reached contradictory conclusions regarding the prognostic value of survivin nuclear expression (18). Since its discovery in 1997 (23), five previous studies have investigated the immunohistochemical status of survivin in urothelial bladder cancer (25-28). Only that of Lehner *et al*. (26) evaluated the cytoplasmic and nuclear expression of survivin separately. In the other studies, this crucial parameter was ignored, though acknowledged as a potential limitation in the Shariat *et al*. study (27). In the present study, positive nuclear and cytoplasmic expression of survivin was detected in 67% and 22% of all the cases, respectively. The nuclear value was within the range of the previously published results, which have nevertheless provided variable expression rates for survivin from 47% to 78%. In the present study, the survivin staining pattern correlated strongly with the tumor grade and stage, partly reaffirming the reported findings of Swana *et al*. (28) and Shariat *et al*. (27). Lehner *et al*. (26) failed to reveal any association of nuclear staining with tumor grade and stage. They also supported the view that the presence of nuclear localization correlated with a greater period of disease-free survival, compared to the patients with urothelial bladder cancer that showed no nuclear staining. These findings were entirely opposite to the present conclusions. Lehner *et al*. detected nonspecific staining in normal bladder mucosa, which suggested low reliability of polyclonal antibody applied in their study, as explained above. Based on the present results, nuclear localization correlated with worse disease-free survival compared to that of the patients whose tumors showed no nuclear staining. In the multivariate analysis, nuclear survivin expression was a significant independent indicator of disease recurrence (*p*-value=0.016).

Survivin is a potential molecular marker for prediction of bladder cancer behavior, but further validation in a larger prospective series is required.

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