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Expression and prognostic significance of *LIVIN*, *SURVIVIN* and other apoptosis-related genes in the progression of superficial bladder cancer

P. Gazzaniga[1], A. Gradilone[1], L. Giuliani[1], O. Gandini[1], I. Silvestri[1], I. Nofroni[1], G. Saccani[2], L. Frati[1] & A. M. Aglianò[1]*

[1]*Dipartimento di Medicina Sperimentale e Patologia, Università degli Studi di Roma 'La Sapienza', Rome;* [2]*Dipartimento di Patologia e Medicina di Laboratorio, Università degli Studi di Parma, Parma, Italy*

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Background: It has been suggested that progression of superficial bladder cancer may be regulated at the molecular level by a typical pattern of expression of genes involved in apoptosis. Recently LIVIN, belonging to the inhibitors of apoptosis (IAP) family, has been found to be expressed in most solid tumors, where its expression is suggested to have prognostic significance. No data are available concerning the significance of LIVIN in the progression of bladder tumors.

Patients and methods: In the present paper we used RT–PCR to investigate the expression of *LIVIN* isoforms α and β, *SURVIVIN*, *BCL-X* and *BCL-2*/*BAX* expression ratio both in normal and tumoral bladder tissues, and correlated their expression with the emergence of early relapses in a follow-up of 4 years. This study shows that only the α isoform of *LIVIN*, which is not expressed in normal bladder tissue, is expressed in a proportion of tumors with a high risk of relapse.

Results: *LIVIN* was found in 7/30 patients (23%), *SURVIVIN* in 9/30 (30%), *BCL-2*/*BAX* ratio >1 in 16/30 (53%), *BCL-2*/*BAX* expression ratio <1 in 14/30 (46.6%) and *BCL-X*, only in isoform *BCL-X*$_L$, in 11/30 (36.6%). When we evaluated the dependence between each gene expression and relapse free time of patients, we found that *LIVIN*, high *BCL-2*/*BAX* ratio and *BCL-X*$_L$, but not *SURVIVIN*, reached statistical significance in order to predict relapses.

Conclusions: Our findings suggest that *LIVIN* may be involved in the progression of superficial bladder cancer and used as a marker of early recurrence; while the expression of *SURVIVIN* cannot be used to identify patients with high risk of relapse.

Key words: *BCL-2*/*BAX*, *BCL-X*, bladder cancer, *LIVIN*, relapse, *SURVIVIN*

Introduction

It has been claimed that superficial tumors of the urinary bladder represent a disease with variable clinical behavior, which shows a clear tendency to early relapse in almost 60% of patients, independent of clinical prognostic variables [1]. An increasing number of studies have focused on the identification of urinary and circulating tumor markers that may represent an adjunct to traditional diagnostic techniques [2]. It has been also suggested that these markers may provide information allowing the appropriate selection of candidate patients for adjuvant therapy. The progression of superficial bladder cancer is known to be regulated at the molecular level by the amplification or rearrangement of some genes; in this context *c-erbB1*, *c-erbB2*, *p53* and c-*myc* are thought to be involved [3]. Nevertheless, these markers seem unable to predict the outcome of disease. In recent years, the hypothesis that altered pathways of cell death may contribute to the progression of disease has been put forward. After the first report on *BCL-2* gene expression in a proportion of bladder tumors [4], the expression ratio between *BCL-2* and *BAX*, the first inhibiting and the second inducing apoptotic cell death, has been found to determine the potential of cancer progression and in part predict the clinical progression of superficial disease [5]. Recently, novel proteins which suppress apoptosis through caspase-dependent and caspase-independent mechanisms have been characterized, and named inhibitors of apoptosis (IAPs). In humans, six members of the IAP family have been described: HIAP1, HIAP2, XIAP, NIAP, *SURVIVIN* and, more recently, *LIVIN*. Two splicing variants have been described for *LIVIN*, which are almost identical except for a 54 bp truncation in exon 6. Despite their similarity, the two isoforms, termed α and β, have been shown to have different antiapoptotic properties *in vitro* [6]. In fact, while both the α and β isoforms seem to block apoptosis

**Correspondence to:* Professor Anna Maria Aglianò, Dipartimento di Medicina Sperimentale e Patologia, Viale Regina Elena, 324-00161 Rome, Italy. Tel: +39-06-49973011; Fax: +39-06-4454820;
E-mail: annamaria.agliano@uniroma1.it

induced by TNF-α and anti-CD45 antibody, *LIVIN* β, but not α, has a strong proapoptotic effect after treatment with etoposide.

Detection of *LIVIN* β in fetal tissues, and in placenta in particular, seems to indicate that it may play a role during fetal development. In adult tissues, high expression of both *LIVIN* α and β were detected in heart, placenta, spleen, ovary and lung, while low levels of just *LIVIN* α were found in lymphocytes, brain and skeletal muscle. Furthermore, high levels of both *LIVIN* α and β were detected in melanoma, colon cancer and prostate cancer cell lines. In addition, high levels of just isoform α were detected in some melanoma and lymphoma cell lines. Although extensive studies have been performed concerning the expression of *LIVIN* in most human tissues, no data were available in the literature concerning the expression of *LIVIN* isoforms in bladder tissues and in bladder cancer.

Within the IAP family *SURVIVIN* has also been recently described. Despite its limited expression in normal tissues, *SURVIVIN* seems overexpressed in a variety of human tumors, including breast, colon, pancreas and prostate carcinoma, neuroblastoma, melanoma and non- Hodgkin's lymphoma [7]. Studies performed by immunohistochemistry described presence of *SURVIVIN* in a variable percentage of tumors, ranging from 30% of gastric cancers to 90% of melanomas [8, 9]. Most of these studies found a positive correlation between *SURVIVIN* expression and prognosis of disease, which is more evident in neuroblastoma and in colorectal cancer, where a multivariate statistical analysis revealed that *SURVIVIN* expression is an independent prognostic factor for disease progression [10].

Expression of *SURVIVIN* has been evaluated in bladder cancer by immunohistochemistry and found to be associated with short disease-free interval [11]. Furthermore, *SURVIVIN* has been detected in the urine of patients with bladder cancer, leading to the hypothesis that it could be used as a marker of early diagnosis [12].

The first goal of this study was to evaluate the expression of *LIVIN* isoforms in 24 normal bladder tissues as well as in 30 primary superficial bladder cancer specimens. The same tissue samples were also analyzed for the presence of *SURVIVIN*, *BCL-2*, *BAX* and *BCL-X* mRNA. Furthermore, we evaluated dependence between expression of all these genes and relapse-free time of patients in 4 years of follow-up.

Patients and methods

Patients and tumoral samples

This study included a total of 30 patients affected by primary superficial transitional cell carcinoma of the bladder, who underwent transurethral resection (TUR-B) between January 1996 and January 1998.

The post-surgical pathological stage of each tumor was classified according to the revised tumor–node–metastasis (TNM) staging system. Tumor extension limited to the mucosa (pTa), or the lamina propria (pT1) of the bladder wall was defined as superficial.

Tumor specimens were immediately frozen in liquid nitrogen after surgery and stored at –80°C until use. Informed consent was obtained from all patients.

All patients were subjected to periodical follow-up studies. Patients at high risk for relapse, with multiple lesions or high tumor grade, were treated by TUR-B followed by mitomycin C or BCG. The schedule of the treatment post-TUR was one instillation/week for 8 weeks followed by one instillation/month.

These patients were followed every 3 months with urinary cytology and pelvic ultrasonography for a period of 1 year. If no recurrence was observed during this period, patients were then followed every 4 months for the second year, and every 6 months thereafter.

The mean follow-up period for the study was 39 months (27–51 months). Clinical features of patients are summarized in Table 1.

In addition, since no data were available concerning *LIVIN* expression in bladder tissue, we evaluated the presence of *LIVIN* isoforms α and β in 24 normal bladder tissues, 18 adjacent to the tumor and six taken from autoptic procedures. The same samples were used as controls for the expression of *BCL-2*, *BAX*, *BCL-X* and *SURVIVIN*.

RT–PCR

Total RNA (1 μg) extracted from frozen tissues was reverse transcribed in a final volume of 20 μl with 100 pmol of random examer and 50 U MuLV reverse transcriptase (Perkin Elmer Cetus, Norwalk, CT, USA), according to the manufacturer's guidelines.

Aliquots corresponding to 100 ng RNA were then amplified in PCR buffer containing 25 pmol each primer and 1 U *Taq* polymerase in a final volume of 50 μl.

Aliquots of the same cDNA were amplified with β-actin, *LIVIN*, *SURVIVIN*, *BCL-2*, *BAX* and *BCL-X* primers. Each amplification, except for *LIVIN*, was performed for 30 cycles; a cycle profile consisted of denaturation at 94°C for 30 s, annealing at 60°C (62°C for *BAX* and *BCL-X*) for 30 s and extension at 72°C for 30 s. A sample without RNA was included in each RT–PCR as a negative control; for positive controls, RNA extracted from M14 cell line for *SURVIVIN*, HeLa cell line for *BCL-2* and CaSki cell line for *BAX* and *BCL-X* were used. *LIVIN* primer sequences and amplification conditions were as described [6].

Sequences of the other primers used are as follows: β-actin upstream, 5′-TTAGCTGTGCTCGCGCTACTCTCTC-3′; β-actin downstream, 5′-GTCGGATTGATGAAACCCAGACACA-3′; *SURVIVIN* upstream, 5′-CAGATTTGAATCGCGGGACCC-3′; *SURVIVIN* downstream, 5′- CCAAGTCTGGCTCGTTCTCAG-3′; *BCL-2* upstream, 5′-GTGGAGGAGCTCTTCAGGGA-3′; *BCL-2* downstream, 5′-AGGCACCCAGGGTGATGCAA-3′; *BAX* upstream, 5′-GGCCCACCAGCTCTGAGCAGA-3′; *BAX* downstream, 5′-GCCACGTGGGCGGTCCCAAAGT-3′; *BCL-X* upstream, 5′-TTGGACAATGGACTGGTTGA-3′; *BCL-X* downstream, 5′-GTAGAGTGGATGGTCAGTG-3′. The primers for *BCL-X* were designed to recognize both isoforms, *BCL-X*$_L$ and *BCL-X*$_S$.

The size of the amplified products were 168, 206, 304, 479, 780 and 591 bp for β-actin, *SURVIVIN*, *BCL-2*, *BAX*, *BCL-X*$_L$ and *BCL-X*$_S$, respectively. The size of *LIVIN* isoforms α and β were 368 and 314 bp, respectively.

We then performed a semi-quantitative analysis of *BCL-2*/*BAX* ratio in each sample, as described [5]. In order to verify the specificity of the amplified products, we performed a hybridization with specific oligonucleotide probes, as previously described [13].

Statistical analysis

Statistical analysis was performed using BMDP statistical software, version 7 [14].

Time to first recurrence was calculated from the date of initial surgery and used as the endpoint of the study. Relapse-free time was estimated using the product-limit method of Kaplan–Meier; the difference between the curves was evaluated using the log-rank test (Mantel–Cox method). Chi-square test was used in order to assess the association between expression of *LIVIN*, *SURVIVIN*, *BCL-X* and *BCL-2*/*BAX* ratio and clinical prognostic variables,

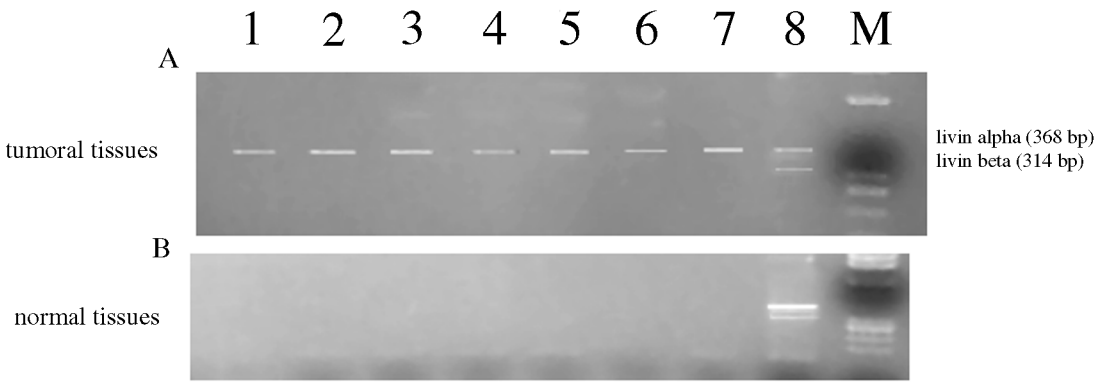

Figure 1. Expression of *LIVIN* isoforms α and β in neoplastic and normal bladder tissues. (**A**) Lanes 1–7, bladder cancer specimens; lane 8, positive control (M14 cell line); (**B**) lanes 1–7, normal bladder tissues; lane 8, positive control (M14 cell line).

such as stage, grade, age and multicentricity of tumors. A value of $P < 0.05$ was considered statistically significant.

Results

In the present study for the first time we evaluated by RT–PCR the presence of *LIVIN* expression in 24 normal and in 30 cancerous bladder tissues. In normal tissues, no detectable levels of either mRNA isoforms were detected. Among tumor tissues, 7/30 (23%) showed expression of isoform α, while none had detectable isoform β expression (Figure 1). *LIVIN* expression does not correlate with any of the known prognostic variables, such as stage, grade and multicentricity.

Moreover, in the same patients *SURVIVIN*, *BCL-2*, *BAX* and *BCL-X* expression was analyzed. Because the experimental evidence suggests that the balance between antiapoptotic and proapoptotic members of the *BCL-2* family is a much better determinant of the sensitivity to apoptosis, we evaluated the *BCL-2*/*BAX* expression ratio, instead of the expression of individual members. In normal tissues, while *BCL-2* and *SURVIVIN* were not found to be expressed, *BAX* was present in 100% and *BCL-X* in 37% of samples (data not shown).

In tumor samples the presence of *SURVIVIN* expression was found in 9/30 (30%) patients, *BCL-2*/*BAX* ratio >1 in 16/30 (53%) and *BCL-X* expression in 11/30 (36%) (Table 1). *BCL-X* gene was found to be expressed only in the *BCL-*X_L isoform (Figure 2).

When we evaluated the dependence between *SURVIVIN* expression and known prognostic variables, we found that *SURVIVIN* expression segregates well with histological grade (5% in G1 versus 89% in G2; $P < 0.05$), but not to clinical stage or multicentricity of the tumors. On the contrary, our analysis showed that higher *BCL-2* levels segregates well only with multicentricity (92% in multicentric forms versus 18% in solitary; $P < 0.05$), but not with clinical stage and histological grade of the tumors. *BCL-*X_L expression segregates well with multicentricity, but not with stage or grade of the tumors (Table 2).

When we evaluated dependence between each gene expression and relapse-free time of patients in the first 4 years of follow-up (mean follow-up period 39 months), we found that *LIVIN* expression, high *BCL-2*/*BAX* expression ratio and *BCL-*X_L, but not *SURVIVIN* expression, reached statistical significance in the prediction of relapses. For instance, median relapse-free time of *LIVIN*-positive patients was 3.5 versus 27.2 months in *LIVIN*-negative patients ($P < 0.0001$). When *BCL-2*/*BAX* ratio was taken into account, patients with a high *BCL-2*/*BAX* ratio had significantly shorter recurrence-free intervals than did patients with a low *BCL-2*/*BAX* ratio. In fact, mean period of relapse-free time of patients with a high *BCL-2*/*BAX* expression ratio was 3 months compared with 48 months for those with a *BCL-2*/*BAX* expression ratio <1 ($P < 0.0001$). In patients with a *BCL-2*/*BAX* expression ratio <1 the percentage of patients relapse-free was 100%, compared to 0% observed in those with *BCL-2*/*BAX* ratio >1. Moreover, the outcome in patients with *BCL-*X_L expression was worse compared with those with no *BCL-*X_L expression. Regarding *BCL-*X_L, the percentage of relapse-free patients was 0% in the group with *BCL-*X_L expression, compared with 73.7% observed in the group with no *BCL-*X_L expression ($P < 0.0005$). In contrast, *SURVIVIN* expression does not correlate with the emergence of relapses ($P > 0.0005$). In patients who relapsed during treatment (mitomycin C or BCG) we found 100% were positive for BCL-2, 66% for BCL-X, 33% for LIVIN, and 16% for SURVIVIN. Results of the survival distribution evaluated by the Kaplan–Meier method are summarized in Table 3.

Discussion

The main purpose of this study was to investigate the expression of some genes involved in apoptotic pathways in neoplastic tissues of patients affected by superficial bladder cancer, and to correlate their expression to the emergence of relapses. The most interesting data was the finding that *LIVIN*, a new member of the IAP family, was found to be not expressed in any of the normal bladder tissues, and expressed only as the isoform α in a proportion of superficial bladder cancer patients (23%). *LIVIN* has been described in two isoforms, α and β, showing different antiapoptotic properties *in vitro*. In fact, while both isoforms are involved in blocking apoptosis induced by TNF-α and anti-CD95, *LIVIN* β seems to be more effective than *LIVIN* α in blocking apoptosis induced by DNA damaging agents, such as etoposide [6].

Table 1. Clinical, pathological and molecular features of superficial bladder cancer patients

Patients	Age	Stage	Grade	Multicentricity	Treatment	*LIVIN* expression	*SURVIVIN* expression	*BCL-2/BAX* expression ratio	*BCL-X* expression	Relapse-free time (months)
1	67	I	1	Yes	Mit	neg	neg	>1	neg	3
2	64	I	1	No	/	neg	neg	<1	neg	48[a]
3	49	0	1	No	/	neg	neg	<1	neg	48[a]
4	65	I	1	No	/	neg	neg	<1	neg	48[a]
5	72	I	1	Yes	BCG	neg	neg	>1	pos	6
6	66	0	1	No	/	neg	neg	<1	neg	48[a]
7	45	I	1	No	/	pos	neg	>1	pos	3
8	46	I	1	Yes	Mit	neg	neg	>1	pos	6
9	74	I	2	Yes	Mit	pos	pos	>1	pos	6
10	70	0	2	No	Mit	neg	pos	<1	neg	48[a]
11	43	I	1	No	/	pos	pos	>1	pos	3
12	52	I	2	Yes	Mit	neg	pos	>1	neg	3
13	54	I	2	Yes	BCG	pos	pos	>1	pos	3
14	70	I	2	No	Mit	neg	pos	<1	neg	36[a]
15	48	I	1	No	/	neg	neg	<1	neg	24[a]
16	27	I	2	No	BCG	neg	pos	<1	neg	48[a]
17	66	I	2	No	Mit	neg	pos	<1	neg	48[a]
18	71	I	1	No	/	neg	neg	<1	neg	48[a]
19	73	0	1	Yes	Mit	pos	neg	>1	pos	6
20	48	0	1	No	/	neg	neg	<1	neg	48[a]
21	85	I	1	Yes	Mit	pos	neg	>1	pos	3
22	55	I	1	No	/	neg	neg	<1	neg	36[a]
23	56	I	2	No	BCG	neg	pos	<1	neg	36[a]
24	63	0	1	No	/	neg	neg	<1	neg	48[a]
25	65	I	1	Yes	BCG	pos	neg	>1	pos	3
26	27	0	1	Yes	Mit	neg	neg	>1	pos	3
27	76	I	1	No	/	neg	neg	>1	neg	3
28	67	0	1	Yes	BCG	neg	neg	>1	pos	6
29	76	0	1	Yes	BCG	neg	neg	>1	neg	3
30	73	I	2	Yes	Mit	neg	neg	>1	neg	3

[a]Duration of follow-up.
Mit, mitomycin C.

Tissue distribution of *LIVIN* has been recently described: elevated levels of both *LIVIN* isoforms α and β have been detected in heart, placenta, lung, spleen and ovary, while *LIVIN* β alone has been detected specifically in fetal tissues. Adult kidney seems to express only isoform β. *LIVIN* α alone has been detected in brain, skeletal muscle and peripheral blood lymphocytes.

Since no data were available concerning *LIVIN* expression in bladder, our study demonstrates for the first time that expression of both *LIVIN* isoforms α and β are not detectable in normal bladder tissues, as previously described for *BCL-2*, a gene with antiapoptotic properties.

Furthermore, while *LIVIN* expression was detected in a variety of cancerous cell lines, no data were available concerning the expression of *LIVIN* isoforms in tumor tissues. Our study for the first time demonstrates that *LIVIN* isoform α is expressed in a proportion of superficial bladder tumors, all of which had a clear tendency to early relapse, showing an unexpected short relapse-free time. Data from the literature have demonstrated that, whether both *LIVIN* isoforms are involved in blocking apoptosis induced by anti-CD95 and TNF-α, isoform α is less efficient than isoform β in blocking apoptosis induced by DNA damaging agents. Nevertheless, it is conceivable that in bladder tumors the expression of *LIVIN* isoform α, together with an overexpression of *BCL-2* upon *BAX*, may be sufficient to give cells a strong resistance to chemotherapy-induced apoptosis.

In the same group of tumor samples we investigated the presence of *SURVIVIN*, another member of the IAP family, which other authors described as being absent in normal bladder

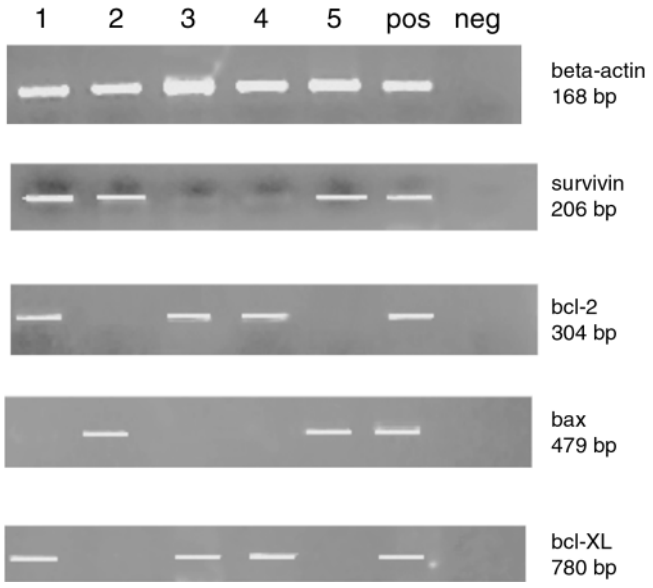

Figure 2. Expression of *SURVIVIN*, *BCL-2*, *BAX* and *BCL-X* in superficial bladder cancer. Agarose gel electrophoresis of RT–PCR products: lanes 1–5, bladder cancer specimens. Positive control: RNA from M14 cell line for β-actin and *SURVIVIN*; RNA from HeLa cells for *BCL-2*; RNA from Caski cell line for *BAX* and *BCL-X* amplification. Negative control: sample with all the reagents except template.

tissue and expressed in bladder tumors, and other genes known to be involved in the pathogenesis of bladder cancer, specifically *BCL-2*, *BAX* and *BCL-X*. *LIVIN* expression does not seem to be correlated with known prognostic variables, such as grade, stage and multicentricity; furthermore, no significant association was found between *LIVIN* and *SURVIVIN* expression, or between *LIVIN* and *BCL-2* or *BCL-X* expression. These results may reflect the different transcriptional pathways of these genes.

Follow-up data obtained in the first 4 years after surgery revealed that patients whose tumors showed expression of *LIVIN* isoform α had a very short relapse-free time (3.5 months), similar to that observed in patients whose tumors expressed a high *BCL-2*/*BAX* ratio. In all patients who relapsed during the course of treatment we found a high *BCL-2*/*BAX* expression ratio. This further stresses the role of high BCL-2 expression as a mechanism by which tumor cells escape chemotherapy-induced apoptosis.

On the contrary, *SURVIVIN* expression in our samples does not correlate with the emergence of early relapse, although its expression seems to be associated with histological grade of tumors.

The only data available concerning *SURVIVIN* in bladder tumors seem to indicate that *SURVIVIN*, but not *BCL-2*, detected by immunohistochemical methods, is significantly associated with time of recurrence and histological grade [11]. We cannot really explain the discrepancy between the data of Swana et al. [11] and ours, mainly due to the lack of sufficient clinical data in that series of patients (size of tumors, modalities of treatment after surgery). Moreover, it is known that *BCL-2* alone in bladder cancer, as well as in other solid malignancies, is not sufficient to predict disease recurrence. Thus, the lack of association between

Table 2. Dependence between clinicopathological features and gene expression (chi-square test)

Clinicopathological features	*LIVIN* expression (%)	*P* value	*BCL-2*/*BAX* expression ratio >1 (%)	*P* value	*SURVIVIN* expression (%)	*P* value	*BCL-X*$_L$ expression (%)	*P* value
Stage								
0	11		44		11		44	
I	55	0.1[a]	56	0.48[a]	38	0.43[a]	38	0.5[a]
Grade								
1	23		62		5		42	
2	11	0.38[a]	44	0.32[a]	89	0.001	22	0.3[a]
Multicentricity								
Yes	30		92		23		69	
No	11	0.4[a]	18	0.001	35	0.4[a]	11	0.03

[a]Not significant.

Table 3. Estimation of relapse-free time by the Kaplan–Meier method[a]

	Multicentricity		*BCL-2/BAX* expression ratio		*BCL-X*$_L$ expression		*LIVIN* expression	
	Yes	No	<1	>1	Pos	Neg	Pos	Neg
Mean relapse-free time (months)	3	36	48	3	3	36	3.5	25.8
% free	0	82.4	100.0	0	0	73.7	0	58.4
Mantel–Cox test	*P* <0.0001		*P* <0.0001		*P* <0.0005		*P* <0.005	

[a]The difference between the two curves was investigated by the log-rank test (Mantel–Cox method).

BCL-2 expression and poor prognosis in that series of patients may be due to altered expression levels of other family members, like *BAX*, whose presence was not investigated by Swana et al. [11].

The emergence of early relapses in patients with high levels of *BCL-2* expression may also depend on the known ability of *BCL-2* to inhibit chemotherapy-induced apoptosis, as previously described in bladder, lung and breast cancer [15–17]. It is thus conceivable that *SURVIVIN* does not share with *BCL-2* the ability to block mitomycin-C-induced apoptosis. In fact, few data are available concerning the efficacy of *SURVIVIN* in blocking chemotherapy-induced apoptosis. To date, *SURVIVIN* has been shown to inhibit apoptosis of NIH3T3 transfectants induced by paclitaxel, and apoptosis of 293 cell line induced by etoposide, but it seems ineffective against microtubule depolymerizing agents such as vincristine [18]. Due to the small number of patients whose results were positive for *LIVIN*, it is still difficult to establish whether *LIVIN* expression may interfere with chemotherapy-induced apoptosis.

Unlike other solid malignancies, such as breast, gastric and colorectal cancer, the presence of *SURVIVIN* in our series of bladder tumor specimens does not seem to be associated with high levels of *BCL-2*. This seems unsurprising, due to the fact that *SURVIVIN* and *BCL-2* do not completely share common mechanisms of transcriptional activation [6]. In addition, similar to what has been observed in other tissues, no correlation was found between *LIVIN* isoform α and *SURVIVIN* expression in bladder cancer.

The emergence of local relapses in superficial bladder cancer represents one of the major problems in the clinical management of this tumor. In fact, it is widely acknowledged that stage and grade are often unable to predict the local progression of Ta–T1 low grade bladder tumors [1], and that 50% of patients affected by superficial disease have local recurrence in the first year of follow-up. This is the reason why in 1996 the European Organization for Research and Treatment of Cancer and Medical Research Council defined the utility of prophylactic treatment also in stage Ta–T1–G1 bladder cancer patients, since it gives a clear advantage in terms of duration of disease-free interval [19]. In view of this, the search for a panel of molecular markers could be useful in the identification of patients with a higher risk of relapse. In our series of patients, the combination of a high *BCL-2*/*BAX* ratio, high *BCL-X* expression and *LIVIN* isoform α seems to identify a subset of patients with shorter relapse-free time.

Our findings, which suggest for the first time a role of *LIVIN* isoform α in the progression of superficial bladder cancer, also indicate that the expression of *SURVIVIN* cannot be used to identify patients with a higher risk of early relapse.

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