

Expression of Survivin and Its Spliced Variants in Bladder Tumors as a Potential Prognostic Marker

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Introduction: Survivin, a novel inhibitor of apoptosis, is re-expressed in a vast majority of human cancers and is widely considered as a diagnostic marker of cancers. Survivin protein regulates both cell division and apoptosis. There are at least 5 spliced variants of the gene with different subcellular localization and anti-apoptotic property. We examined the expression pattern of survivin and its 2 spliced variants, survivin- Δ Ex3 and survivin-2B, and their prognostic values in archival collections of formalin-fixed paraffin-embedded samples of bladder tumors.

Materials and Methods: Total RNA from formalin-fixed paraffin-embedded samples (51 samples from 30 patients with bladder cancer and 5-year follow-up) were extracted and analyzed by semi-quantitative reverse transcriptase polymerase chain reaction technique. Tissue distribution and subcellular localization of survivin protein in tumor tissues was also examined by immunohistochemistry.

Results: The expression of survivin, survivin- Δ Ex3, and survivin-2B were detected in 66.6%, 47.8%, and 54.7% of the specimens, respectively. The expression of survivin and survivin- Δ Ex3 were preferentially elevated in tumors with higher grades, whereas survivin-2B expression was lower in high-grade tumors ($P = .04$). A reverse correlation was observed between survivin-2B expression and high-grade tumors. Immunohistochemistry results also confirmed the nuclear localization of survivin protein within tumoral cells.

Conclusion: We were successful in detecting the expression of survivin and its variants in formalin-fixed paraffin-embedded bladder samples. Furthermore, our results showed that overexpression of survivin and survivin- Δ Ex3 in bladder tumors correlates with poor prognosis of bladder cancer. We suggest that survivin and its variants are suitable prognostic markers of bladder tumors.

Keywords: survivin protein, apoptosis, bladder neoplasms, reverse transcriptase polymerase chain reaction

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INTRODUCTION

Similar to other types of tumors, bladder cancer initiates and progresses from genetics and epigenetic events affecting the delicate regulations imposed on cell proliferation and programmed cell death (apoptosis).⁽¹⁾ A tightly regulated balance between the presence of pro-apoptotic and anti-apoptotic regulators would

determine the survival/death fate of each cell. Deregulated expression of inhibitors of apoptosis may lead to the prolonged survival of transformed cells, and hence, tumor initiation, progression, or metastasis.^(2,3) There are 2 major protein families involved in regulating the rate of apoptosis: B-cell CLL/lymphoma 2 protein and inhibitors of apoptosis proteins

(IAPs).^(4,6) The IAPs are a group of evolutionary conserved proteins characterized by the presence of 1 to 3 domains known as baculoviral IAP repeat domains, which are believed to be responsible for the anti-apoptotic function of the IAPs.⁽⁵⁾

Survivin, a unique member of the IAP family, is known to be involved in both regulation of apoptosis and control of cell division.⁽⁷⁾ It is highly expressed during normal tissue development, but is absent in most terminally differentiated cells of adult tissues. Recent identification of several functionally divergent survivin variants in mouse and human increases the complexity of survivin action as well as its regulation. At least, 5 different spliced variants of survivin have been reported so far in human.^(8,9) Loss of exon 3 in survivin- Δ Ex3 results in a truncated baculoviral IAP repeat domain and a frameshift of the COOH-terminal. In survivin-2B, partly retention of intron 2, as a cryptic exon, inserts 23 additional amino acids into the baculoviral IAP repeat domain at essentially the same position.⁽⁸⁾ Despite these profound structural alterations, the anti-apoptotic potential of survivin- Δ Ex3 was largely preserved, while survivin-2B exhibited a loss of anti-apoptotic potential. The overexpression of survivin and survivin- Δ Ex3 in almost all human malignancies, and low or no expression in most normal tissues, suggests that it could be a good diagnostic and prognostic marker of cancers as well as an ideal target for cancer-directed therapy.^(10,11)

Considering the necessity of using new molecular markers for assessing prognosis of bladder tumors, and also because of the potential application of survivin and its variants as specific tumor markers for cancers in general, we evaluated the prognostic value of expression of survivin and its two major variants, survivin- Δ Ex3 and survivin-2B, in formalin-fixed paraffin-embedded (FFPE) samples of patients with bladder cancer, by means of reverse transcriptase polymerase chain reaction (RT-PCR) and immunohistochemistry techniques.

MATERIALS AND METHODS

Sampling

Human samples were obtained from the FFPE archival collections of Shahid Labbafinejad

Medical Center in Tehran. Tissues with known 5-year follow-up history records were selected for further analysis. All of the FFPE samples were sectioned and deparaffinized with xylene and stained with hematoxylin-eosin, in order to confirm their previously determined stages and grades.

RNA Extraction

A total number of 51 specimens from 30 patients were collected and 3 to 5 sections of each block were prepared for RNA extraction process. The sections were deparaffinized with xylene and alcohol, and the tissues were treated with proteinase K (Fermentase, Vilnius, Lithuania). Then, the pellets were treated with the RNX plus solution (Cinnagen, Tehran, Iran) according to the manufacturer's instructions, as described previously.⁽¹²⁾

Reverse Transcriptase Polymerase Chain Reaction

Specific primers of human beta-2-microglobulin (B2M; as an internal control), human survivin, survivin- Δ Ex3, and survivin-2B were designed using the Gene Runner software, version 3.6 (Hastings Software, Hastings, New York, USA). The sequences of the designed primers and the accession numbers of genes are as follow:

Survivin (accession number: NM_001168.2):

External forward primer:

5'-TGGCAGCCCTTTCTCAAG-3'

External, reverse primer:

5'-GTTCTCTATGGGGTCGTC-3'

These primers amplified a 202-bp segment of human survivin complementary DNA.

Internal, forward primer:

5'-ACCACCGCATCTCTACATTC-3'

Internal, reverse primer:

5'-GAAGAAACACTGGGCCAAG-3'

These primers amplified a 131-bp segment from human survivin complementary DNA.

Survivin- Δ Ex3 (accession number:

NM_001012270.1):

Forward primer:

5'-ATGACGACCCCATGCAAAG-3'

External, reverse primer:

5'-ACAGGAAGGCTGGTGGCAC-3'

These primers amplified a 184-bp segment of human survivin complementary DNA. For a heminested RT-PCR reaction, we used an internal reverse primer with the same forward one.

Internal reverse primer:

5'-CCTGGAAGTGGTGCAGCC-3'

This primer together with the forward primer amplified a 153-bp segment.

Survivin-2B (accession number: NM_001012271.1):

Forward primer:

5'-CGGATCACGAGAGAGGAAC-3'

External, reverse primer:

5'-CTTTCTCCGCAGTTTCCTC-3'

These primers amplified a 181 bp segment of human survivin complementary DNA.

Internal reverse primer:

5'-TTTCTTCTTATTGTTGGTTTCC-3'

This primer together with the forward primer amplified a 156 bp segment.

Beta-2-microglobulin (accession number: NM-012512):

Forward primer:

5'-CTACTCTCTCTTCTGGCCTG-3'

Reverse primer:

5'-GACAAGTCTGAATGCTCCAC-3'

The product of amplification with these primers was a 191-bp segment from human B2M complementary DNA.

The primers were synthesized by MWG-Biotech (Ebersberg, Germany) as high purified salt-free grade. All designed primers were blasted with human genome to make sure they are not complementary to other regions of the genome.⁽¹³⁾ Complementary DNA synthesis reactions were performed using 11- μ L RNA and M-MuLV reverse transcriptase (Fermentas, Vilnius, Lithuania) with random hexamer priming in a 20- μ L reaction, as previously described.⁽¹⁴⁾ Polymerase chain reaction assay was performed using 2 μ L of synthesized complementary

DNA with 0.2 μ L of Taq polymerase (5 U/ μ L, Cinnagen, Tehran, Iran), as described elsewhere.⁽¹⁸⁾

The PCR amplification was performed for 20 to 35 cycles. The cycling conditions were as follows: 94°C for 40 seconds, 57°C for 45 seconds (for survivin external primers; for other primers see the results), 72°C for 1 minute, and a final extension at 72°C for 10 minutes. The PCR products were then separated on a 2% agarose gel and visualized by ethidium bromide staining.

For a semiquantitative comparison of survivin expression among different samples as well as their different recurrences, we used B2M as an internal control. For each sample, the RT-PCR was performed under similar conditions (except for the number of cycles and the annealing temperature, see the results), but in 4 separate tubes for B2M, survivin, survivin- Δ Ex3, and survivin-2B.

The band intensity for each gene was determined with Uvitech software (Cambridge, UK) and the expression levels were measured in relation with the B2M expression level for each sample. The data were statistically analyzed using the SPSS software (Statistical Package for the Social Sciences, version 13.0, SPSS Inc, Chicago, Illinois, USA).

Immunohistochemistry

Using a polyclonal antisurvivin antibody (Novus Biologicals, Littleton, Colorado, USA), we examined the subcellular localization of survivin protein in bladder tissues using immunohistochemistry. Sections of samples with proved survivin-negative and survivin-positive expression at mRNA level were further examined by immunohistochemistry. In these samples, expression or no expression of survivin was confirmed by RT-PCR reaction, according to the expression of B2M. Briefly, the FFPE tissue sections (5 μ m) were deparaffinized with xylene, rehydrated in descending concentrations of ethanol, and boiled for 15 minutes in citrate buffer (9 mM, pH 6.0) for antigen retrieval. Then, endogenous peroxidase activity was suppressed with 3% H₂O₂ for 20 minutes. The slides were serum-blocked (with normal goat serum) and incubated with survivin's primary

antibody (1:500 dilution) at 4°C for overnight. The sections then incubated with the secondary antibody (Vector, Burlingame, California, USA), a biotinylated antirabbit antibody (1:250 dilution), for overnight at 4°C. The sections were then processed and stained with a complex of diaminobenzidine/H₂O₂ (1:1000). In negative control, all the conditions were kept the same, except that the first antibody was eliminated.

RESULTS

Polymerase Chain Reaction Optimization

To find the optimal annealing temperature for each pair of primers, PCR reactions in a gradient of temperatures from 55°C to 65°C were performed for each round of PCR. The optimum annealing temperature for the survivin primers was 57°C and 62°C for the first and second round of PCR reaction, respectively. For survivin-ΔEx3 primers, the annealing temperature was 61°C for both rounds of PCR. Finally, 55°C was determined as the optimum annealing temperature to amplify survivin-2B, for both rounds of PCR (data not shown). The same strategy was used to find the best concentrations of Magnesium chloride (2 mM), to optimize the PCR reactions (data not shown).

Owing to the weak intensity of the signal in the first round of PCR, a second round (nested) of PCR was performed on the product of the first round, using internal primers for 10 to 30 cycles for survivin and 25 to 40 cycles for the survivin variants. For survivin, the optimal number of cycles was determined as 35 and 18 for the first and second round of PCR, respectively. The optimal cycle numbers for survivin-ΔEx3 were 35 and 37 for the first and second round of PCR, respectively. Finally, for amplifying survivin-2B, the number of cycles was 35 for both rounds of PCR reaction. For B2M amplification, the annealing temperature of 57°C and the cycle number of 35 were similarly determined (data not shown).

Differential Expression of Survivin and Its Spliced Variants

Overall, 51 tumor specimens from 30 patients

were studied. Clinicopathological characteristics of all patients were further confirmed by an expert urologist (Table).

To assure that equal amounts of RNA were used for each RT-PCR, we used B2M as an internal control and compromise the relative survivin expression of each sample with its own B2M expression. For each specimen, the RT-PCR was performed under similar conditions within 4 separate tubes, for B2M and for survivin and its variants. As expected, B2M was expressed in all tumor specimens (Figure 1). Furthermore, the nested or heminested RT-PCR results on the same specimens amplified an expected 131-bp band for survivin and 2 bands of 153 bp and 156 bp for survivin-ΔEx3 and survivin-2B, respectively (Figure 1). The experiments were repeated at least twice for all specimens. Overall, expression of survivin was detected in 34 out of 51 tumor tissues (66.7%), while survivin-ΔEx3 and survivin-2B were detected in 11 of 23 (47.8%) and 23 of 42 (54.8%) FFPE tissues, respectively.

After performing RT-PCR for all the samples and determining the band intensity for each gene, statistical analysis of the correlation between the expression of survivin, survivin-ΔEx3, and survivin-2B and different clinical and pathological characteristics of the cases was performed. The

Clinicopathological Features of Patients With Bladder Cancer*

Characteristic	Value
Number of patients	30
Mean age at diagnosis, y	62.4 (31 to 84)
Cystectomy	
Radical	9 (30.0)
Partial	1 (3.3)
Number of specimens	51
Tumor grade at diagnosis	
1	5 (16.7)
2	20 (66.7)
3	5 (16.7)
Tumor stage	
A	29 (96.7)
B	1 (3.3)
Metastasis	8 (26.7)
Follow-up	
No tumor	10 (33.3)
Recurrence	12 (40.0)
Lost to follow up	8 (26.7)
Death	6 (20.0)

*Values in parenthesis are percents, except for age, which is range.

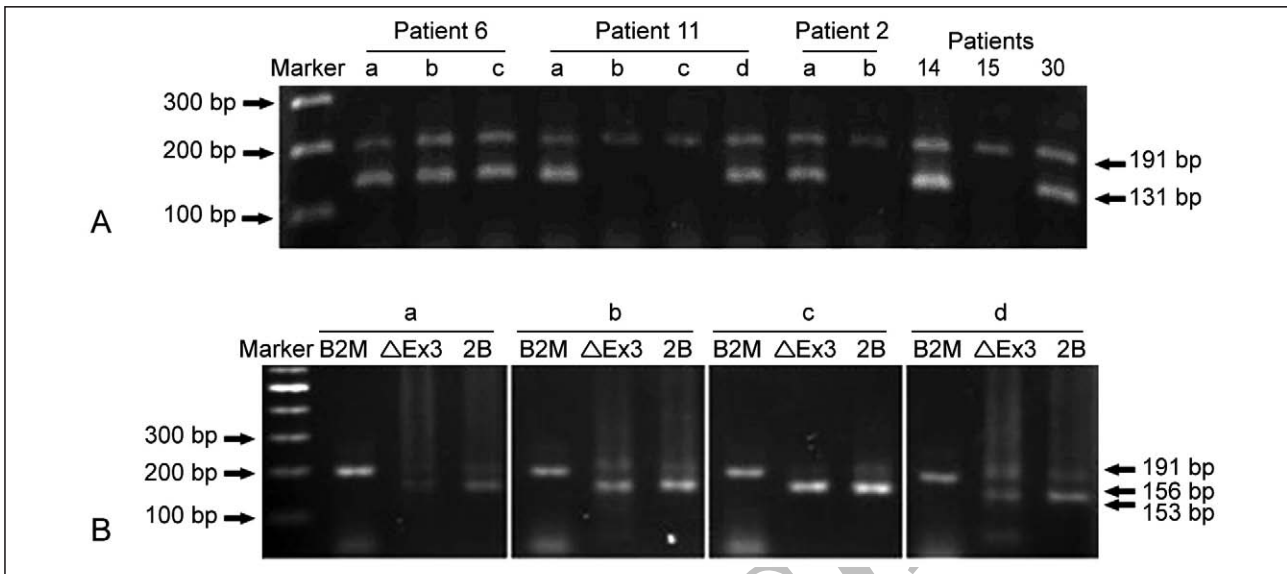


Figure 1. Reverse transcriptase polymerase chain reaction analysis of the expression of (A) B2M (191 bp) and survivin (131 bp), and (B) B2M, survivin-ΔEx3 (153 bp), and survivin-2B (156 bp). The a, b, c, and d indicate subsequent recurrences of the same patient.

samples were classified according to the age and sex of the patients, 5-year survival rate, stage, grade of malignancy, and recurrences. There was a significant correlation between the expression level of survivin and the 5-year survival rate (odds ratio, 9.15; $P = .04$; Figure 2). In low-survivin and high-survivin expression groups (expression above the median was considered as high, and below the median, as low), elevation in survivin expression correlated with a decrease in the survival rates of the patients by 6 times (odds ratio, 6.0). Moreover, our data demonstrated that survivin

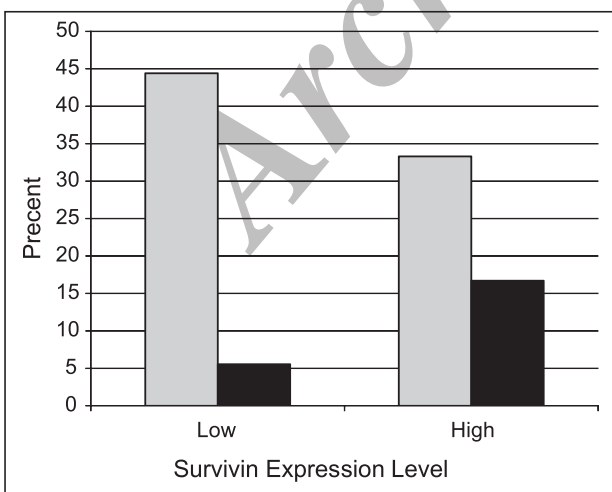


Figure 2. Proportional survivin expression levels (high expression and low expression) in dead and alive patients. Those with high expression level of survivin had lower survival rate compared to the group having low or no expression of survivin.

expression was higher in high-grade tumors; however, the difference was not significant (Spearman rho test; Figure 3).

There was no significant correlation between the expression level of survivin and the stage of the tumors. Elevated expression of survivin-ΔEx3 was observed in higher stages and in the 2nd and 3rd recurrences of the tumor, but this finding was not significant. Furthermore, there was no significant correlation between the relative overexpression of survivin-2B and the survival rate of the patients. However, the data showed that with 1 unit of overexpression, survival rate would increase 2.3

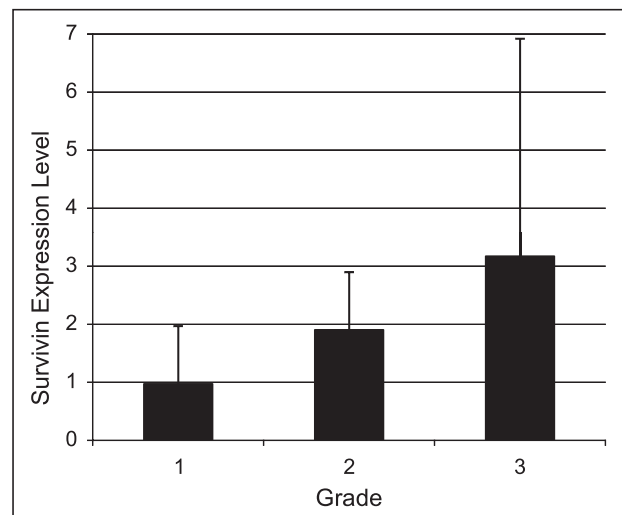


Figure 3. Proportional survivin expression levels in bladder tumors with different grades.

times (odds ratio, 2.3). Our results also revealed that in high-grade tumors, and also in the 2nd and 3rd recurrences of the tumors, survivin-2B was downregulated. However, these observations were not statistically significant.

Identity of Survivin and Its Variants

The identities of the amplified segments of the complementary DNAs corresponding to the survivin, survivin- Δ Ex3 and survivin-2B were confirmed by direct sequencing (data not shown).

Intracellular Localization of Survivin in Bladder Tumors

A survivin-positive sample (according to the RT-PCR results) was used for optimizing the experiment and choosing the required dilution of the primary and secondary antibodies. Then, the immunohistochemistry was performed on tissue sections of 3 survivin-positive and 2 survivin-negative specimens. A specific signal was visualized in the nuclei of the survivin-positive tumor cells, while no immunoreactivity was observed in survivin-negative samples. For confirming the fidelity of immunoreactivity, we also employed negative controls, which were incubated in similar conditions, except for the absence of primary antibody (Figure 4).

DISCUSSION

The main aim of the present study was to examine the potential correlation between the expression of survivin variants and the degree of malignancy, pathological behavior, patient survival rate, and the recurrence of bladder cancer. There are rich reservoirs of FFPE samples for molecular studies in the archive of hospitals and clinical centers. These samples can be used in retrospective studies of patients with cancer and could provide valuable prognostic information for further therapeutic strategies. The routine procedures to study paraffinized samples such as immunohistochemistry provide limited information on the biological nature of tumors. Recent application of PCR and RT-PCR techniques on FFPE samples would provide more accurate diagnostic and prognostic data with higher sensitivity and specificity.⁽¹⁵⁾

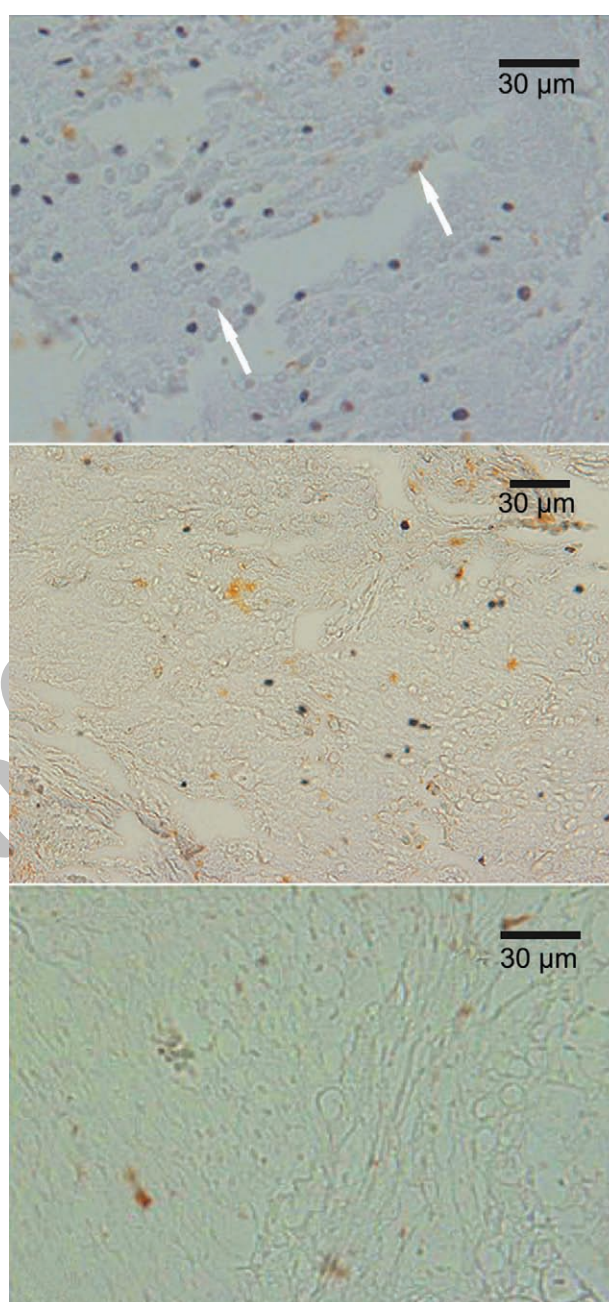


Figure 4. Immunohistochemistry results showing the tissue distribution and subcellular distribution of survivin. **Top,** A survivin-positive specimen (positive in reverse transcriptase polymerase chain reaction). Arrows show the signal within nuclei. **Middle,** A survivin-negative specimen. **Bottom,** a specimen of negative control, processed in the absence of the first antibody.

The current methods for RNA extraction from FFPE samples are achieved by expensive commercial extraction kits, which increase the efficiency of the procedure. In the present study, we were successful to extract reliable RNA from FFPE samples by changing the routine procedures

for RNA extraction from fresh samples. Using this procedure, we were able to demonstrate the expression of survivin in 66.6%, survivin- Δ Ex3 in 47.8%, and survivin-2B in 54.7% of the FFPE specimens. Previous studies on different human tumors such as breast, esophagus, lung, bladder, and gastric tumors have shown a correlation between overexpression of survivin and/or survivin- Δ Ex3 and high stages and grades of the tumor⁽¹⁶⁻²⁰⁾; whereas, a reverse correlation was reported for survivin-2B. Furthermore, the overall survival rate of the patients declines with overexpression of survivin and survivin- Δ Ex3, and these variants are involved in malignant behavior and therapeutic resistance of the tumors.

In accordance with the previous reports,⁽¹⁶⁻²⁰⁾ our study demonstrated that the overexpression of survivin and survivin- Δ Ex3 occurred mostly in tumors with high grades and stages. Furthermore, our results revealed that the death risk of the patients increased with the elevated expression of survivin and survivin- Δ Ex3, and decreased with overexpression of survivin-2B. We failed to document any significant correlations between the levels of expression of survivin variants and other clinicopathological characteristics of the patients, which might be due to our small sample size. Our available data suggest that increasing the number of samples would probably reveal such potential correlations more accurately.

Seven of the 30 patients (23.3%) died and 8 patients (26.7%) had undergone partial or radical cystectomy, and therefore, their specimens were omitted from the group of follow-up. Another problem we faced during this project was the incomplete 5-year follow-up records of the patients. Lack of a precise, uniform, and regular follow-up registry for most of the patients is a challenge for these kinds of research. Precise recording of the patients' information and regular follow-up in hospitals would provide a more reliable reservoir for future retrospective studies.

CONCLUSION

We developed an economical procedure for evaluating the expression of survivin and its splicing variants with no need for expensive kits and techniques. Here, we demonstrated that

overexpression of survivin and survivin- Δ Ex3 correlated with the malignant outcome and recurrence of bladder cancer, while for survivin-2B, a reverse correlation was seen.

CONFLICT OF INTEREST

None declared.

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