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Evaluating Expression and Potential Diagnostic and Prognostic Values of Survivin in Bladder Tumors: A Preliminary Report

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ABSTRACT

Introduction: Survivin, an inhibitor of apoptosis (IAP), has been reported to be capable of regulating both cellular proliferation and apoptotic cell death. Survivin expression has been described during embryonic development and in adult cancerous tissues, with greatly reduced expression in adult normal differentiated tissues, particularly if their proliferation index is low. In the present study, the expression and potential diagnostic and prognostic value of survivin in bladder tumors was evaluated.

Materials and Methods: Primary and recurrent bladder tumor specimens were obtained from patients referred to the Shaheed Labbafinejad Medical Center in Tehran, Iran. Total RNA was isolated from frozen tissues, reverse transcribed and amplified by means of a nested polymerase chain reaction technique.

Results: Survivin was detected in 3 cases of primary tumors (42.8%) and 6 cases of recurrent tumors of bladder (60%). Survivin-ΔEx3 expression was seen in 41.2% of the 17 cases with bladder cancer.

Conclusion: Our findings suggest that the expression of survivin and survivin-ΔEx3 is well associated with invasive and more-aggressive forms of bladder cancer. Our data also indicate that the presence of survivin-ΔEx3 is better correlated with tumorigenesis of bladder cancer compared with survivin expression.

KEY WORDS: survivin, apoptosis, bladder cancer, polymerase chain reaction

Introduction

Bladder cancer is one of the most common malignant tumors worldwide. It is the fourth most-common type of cancer in men and the eighth most-common type in women. The incidence of bladder cancer increases with age, where people older than 70 years develop the disease 2 to 3 times more often than do those aged 55 to 69 years, and 15 to 20 times more often than those aged 30 to 54 years.[1] Despite the fact that there is no comprehensive statistical report on the incidence rate of bladder cancer in Iran, according to clinical reports it has a very high incidence rate, and most cases are transitional cell carcinoma (TCC), similar to that in Europe and North America.

Tumor growth depends on 2 main factors: cell proliferation and cell death by apoptosis.[2] Apoptosis is a form of programmed cell death characterized by morphologic, biologic, and genetic features. Abnormalities of apoptosis may lead to uncontrolled cellular proliferation and ultimately carcinogenesis. Several studies have

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reported significant correlations between apoptosis and prognosis in malignant tumors such as lung, breast, and esophageal cancer.

Two protein families are responsible for controlling apoptosis: BcL-2 and the inhibitors of apoptosis proteins (IAP). Inhibitors of apoptosis proteins are a group of evolutionary conserved proteins characterized by the presence of 1 to 3 domains known as baculoviral IAP repeat (BIR) domains, which is necessary for the antiapoptotic property of IAPs.[3,4]

Survivin, a new member of IAPs, is structurally unique, because it has only a single BIR domain and lacks the COOH-terminal RING finger domain.[5] Survivin also plays critical roles in regulating the cell cycle and mitosis. Its primary expression in most human malignancies and its low or absence of expression in normal tissues suggest that it would be a good diagnostic and prognostic marker as well as an ideal target for cancer-directed therapy.[5,6] Extensive studies have been carried out to elucidate the mechanism of its function; however, its role in regulating cell survival and cell cycle is poorly understood.[7]

Mahotka and colleagues[8] have cloned and characterized 2 novel splice variants of survivin, lacking exon 3 (survivin-ΔEx3) or retaining a part of intron 2, as a cryptic exon (survivin-2B). Both sequence alterations cause marked changes in the structure of the corresponding proteins, including structural modifications of the BIR domain. They also have reported a conservation of antiapoptotic properties for survivin-ΔEx3 and a markedly reduced antiapoptotic potential for survivin-2B. The reduced antiapoptotic activity of survivin-2B is possibly due to a dominant-negative mechanism of competitive binding to the interactive partners of surviving.[9]

Using molecular markers for diagnosing and determining the prognosis of bladder tumors can be of great value. Considering the potential application of survivin as a specific tumor marker for cancers, we decided to evaluate the expression of survivin in bladder tumors using reverse transcription-polymerase chain reaction (RT-PCR) to determine the potential association of its variants with the malignant behavior of the tumor.

Materials and Methods

Specimen Preparation

Tissue specimens were obtained from patients with bladder cancer who had been referred to the Shaheed Labbafinejad Medical Center in Tehran, Iran, from December 2002 to July 2003. The specimens were obtained under the supervision of a urologist and categorized into 2 groups according to their clinical criteria: group 1 consisted of the specimens from patients with newly diagnosed bladder cancer via conventional diagnostic methods such as cystoscopy and urine cytology. Group 2 was composed of known bladder cancer cases that were on follow-up (Table 1).

Tumoral specimens were collected in 2 ways; if the tumor was in stage Ta or T1, low-grade, and noninvasive, the tissue was removed from the suspected area by cystoscopic biopsy. A piece of unused tissue was then put into an RNase-free, 1.5-mL tube, immediately snap frozen in nitrogen vapor, and kept at -80°C until RNA extraction. For the tumors in stages T2-T4, that is, those invading the muscular layer, the whole bladder was removed by radical cystoscopy. A piece of tumoral specimen was then removed and put in an RNase-free tube, snap frozen, and kept at -80°C for later use.

RNA Extraction

Total RNA was isolated from frozen tissues using the RNX-Plus solution (Cinnagen, Tehran, Iran) according to the manufacturer's instructions and as previously described.[10] The purity and integrity of the extracted RNA were evaluated by optical density measurements (260:280 nm ratios) and by visual observation of specimens electrophoresed on agarose gels. Both methods confirmed the integrity of the extracted RNA with little or no protein contamination. After extraction, the isolated RNA was treated with DNase to eliminate a probable genomic DNA contamination.

RT-PCR Reaction

Specific primers of human B2M (β2-microglobulin as an internal control; accession number: NM-004048) and human survivin (accession number: U75285) were designed by using GeneRunner software (Hastings Software, Inc, Hastings, NY, USA). The sequences of the designed primers are as follow:

External, forward primer:

5'- TGGCAGCCCTTTCTCAAG -3'

External, reverse primer:

5'-GAGAGAGAGAAGCAGCCAC - 3'

These primers amplified a 632 bp segment of

TABLE 1. *Patients' characteristics and their clinicopathological conditions*

Groups	Specimens	Age (year)	Sex	Stage	Grade	Pathological diagnosis	Description
Group 1	1	47	M	A/pT1	2	papillary TCC	High mitotic activity, high N/C ratio
	2	68	M	NA	NA	papillary TCC	Multiple nodular mass in bladder
	3	67	F	A/pT1	Low/ small foci of high grade	Carcinoma in situ	High mitotic activity, high N/C ratio
	4	72	M	A/pT1	2	papillary TCC	Primary biopsy diagnosis as carcinoma in situ
	5	59	M	NA	NA	papillary TCC	-
	5	67	F	A	NA	papillary TCC	Rapid recurrent after 4 months of primary diagnosis
	7	60	M	A	2	papillary TCC	Rapid recurrent after 3 months of primary diagnosis
Group 2	1	76	M	NA	NA	papillary TCC	Under follow-up for a long period of time, small tumor
	2	56	M	B2/pT3a	High/3	papillary TCC	High mitotic activity
	3	62	M	NA	Poorly differentiated	Undifferentiated carcinoma	High level of necrosis
	4	46	M	A	Low/2	papillary TCC	Radical cystectomy
	5	67	M	B2/pT3a	Poorly differentiated	SCC	Invading carcinoma with extensive necrosis
	6	67	M	PT2 Nx Mx/B1	High	High-grade invasive urothelial carcinoma	Transition from TCC to SCC
	7	81	M	NA	NA	TCC	-
	8	83	M	pT3b	Low /2	papillary TCC	Invasion to surrounding soft tissues
	9	71	M	pT3b Nx Mx	High /3	TCC	Necrotic tumoral tissue invading lamina propria
	10	81	F	NA	Poorly differentiated	Undifferentiated carcinoma	Presence of diffuse necrosis

M: male, F: female, NA: not available, TCC: transitional cell carcinoma, SCC: squamous cell carcinoma, N/C ratio: nucleus-cytoplasm ratio

human survivin cDNA located between nucleotides 77 and 708.

Internal, forward primer:

5'- ACCACCGCATCTCTACATTC -3'

Internal, reverse primer:

5'- CTGGTGCCACTTTCAAGAC -3'

These primers amplified a 556 bp segment from human survivin cDNA located between nucleotides 96 and 651.

B2M forward primer:

5' TCG CGC TAC TCT CTC TTT CTG 3'

B2M reverse primers:

5' GCT TAC ATG TCT CGA TCC CAC 3'

These primers amplified a 334 bp segment from human B2M cDNA located between nucleotides 41 and 374.

Complementary DNA (cDNA) synthesis reactions were performed using 5 μg RNA and MMLV reverse transcriptase (Gibco BRL, Germany) with oligo (dT)18 priming in a 20 μL reaction as described elsewhere.[11]

The designed primers as well as the oligo (dT)18 primer were synthesized by MWG Biotech Company (Ebersberg, Germany) as highly purified salt-free grade. All designed primers were BLAST[12] compared against the human

genome to make sure they are not complementary with other regions of genome.

PCR was performed using 5 μL of synthesized cDNA with 1.25 U of Taq polymerase (Roche, Germany), as described elsewhere.[10,11] The PCR amplification was performed for 25 to 35 cycles. The cycling conditions were as follows: 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 1 minutes, and a final extension at 72°C for 10 minutes. PCR products were then separated on a 1.5% agarose gel and visualized by ethidium bromide staining.

Restriction Enzyme Digestion

To confirm the identity of PCR products of survivin variants, we determined the restriction size pattern of all amplified products digested with *MboI* restriction enzyme (MBI Fermentas, Hanover, Germany). The enzyme cuts survivin cDNA at nucleotide 588 generating 2 shorter bands that are detected on 8% polyacrylamide gel electrophoresis.

Results

RT-PCR Optimization

To find the optimal number of amplification cycles, external primers were first used to amplify a piece of survivin, which generates a 632-bp segment. A band corresponding to the expected size appeared in the first round of PCR at cycles 35 and 40 (data not shown). Owing to the weak intensity of the signal, nested PCR was performed on the product during the first round of PCR, using internal primers for 25 to 35 cycles. The results showed 2 bands with sizes of 556 and 438 bp, as expected from the sizes previously reported for different variants of the gene (data not shown). For the rest of the experiments, all PCR reactions were performed at 30 cycles for B2M and 35 cycles, first round, and 30 cycles, second round, for survivin.

Evaluating the Expression of Survivin and Its Splice Variants

Overall, 17 tumoral specimens were studied. The ages of the patients were between 46 and 83 years. Demographic and clinicopathological characteristics of all patients are listed in Table 1. To be sure equal amounts of RNA were used in all reactions, we used B2M as an internal control. For each specimen, the RT-PCR was performed under similar conditions (except for the number of cycles) in 2 separate tubes, 1 for B2M and 1 for survivin. B2M was expressed in all tumoral specimens (Figure 1). Nested RT-PCR results on the same specimens revealed an expected band for survivin with a size of 556 bp, as well as another band with the approximate size of 438 bp (Figure 1).

The experiment was repeated at least twice for all specimens. The reappearance of the smaller band in the same specimens points to the potential detection of a survivin splice variant. Changing the PCR conditions (ie, increasing the

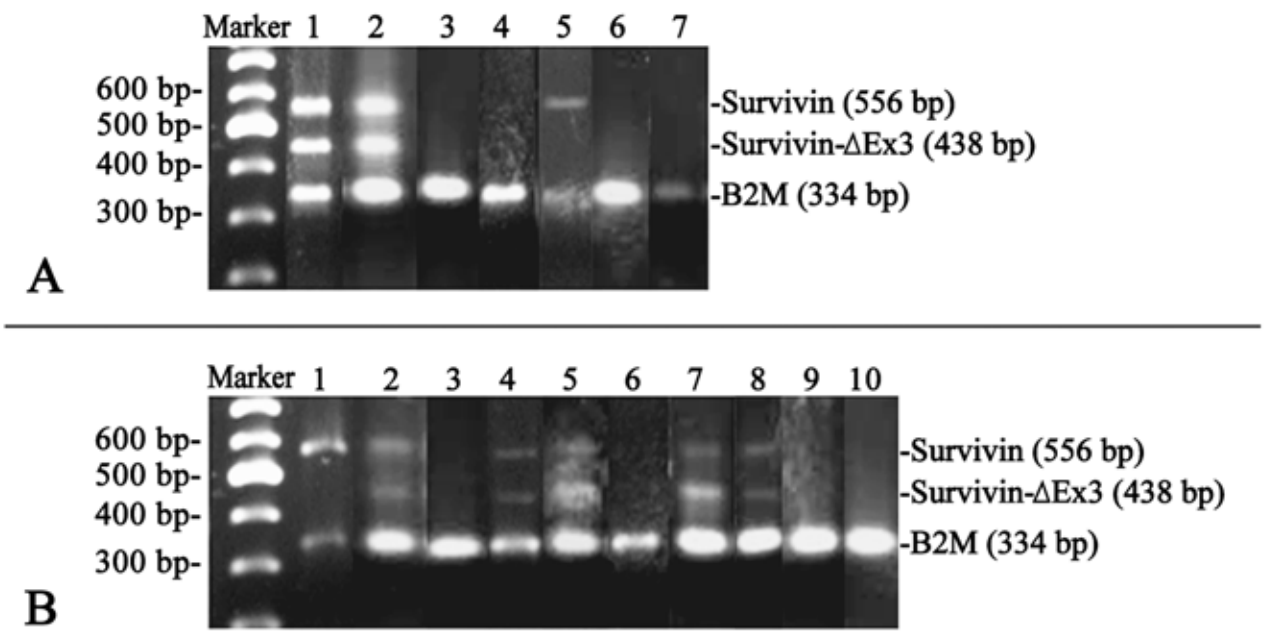

Fig. 1. RT-PCR analysis of the expression of B2M and survivin in tumoral specimens, A. Patients with no previous incidence of bladder cancer, B. Patients with recurrence of bladder cancer.

annealing temperature and/or decreasing the concentrations of the primers) did not affect the intensity of the bands.

In group 1 (specimens from patients with no previous history of bladder cancer), 4 out of 7 cases (specimens 3, 4 , 6, and 7) showed no detectable signal for survivin, while 2 cases (specimens 1 and 2) showed 2 bands with the sizes of 438 and 556 bp, and 1 (specimen 5) had a single 556 bp band (Figure 1A). In group 2 (specimens from patients with previous diagnoses of bladder tumors, currently under follow-up surveillance), 4 out of 10 cases (specimens 3, 6, 9, and 10) showed no signal for survivin. Five cases (specimens 2, 4, 5, 7, and 8) showed 2 bands of 438 and 556 bp, and 1 (specimen 1) had a single 556-bp band (Figure 1B). Overall, survivin was detected in 3 cases of primary tumors (42.8%) and 6 cases of recurrent bladder tumors (60%). Survivin-ΔEx3 expression was seen in 41.2% of the 17 cases with bladder cancer. Survivin-2B was not detected in any of our cases.

Confirming the Identity of Survivin Variants

The identity of the amplified bands in PCR, which are the bands corresponding to the survivin splice variants, was confirmed by means of restriction enzyme digestion. For this purpose, MboI enzyme was applied, which cuts survivin cDNA at nucleotide 588 to generate 2 shorter segments (64 and 492 bp for surviving, and 64 and 374 bp for survivin-ΔEx3; Figure 2).

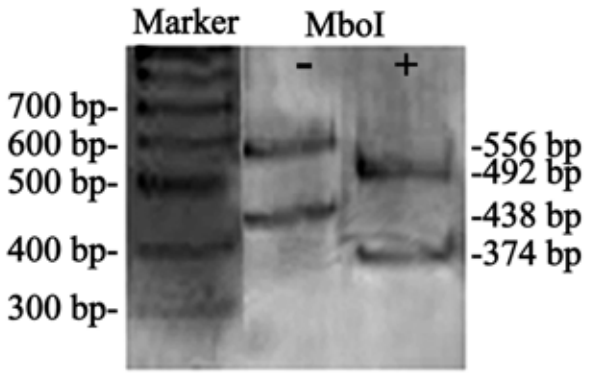

FIG. 2. Electrophoresis of digested products of amplified survivin segments by MboI on 8% polyacrylamide gel. The enzyme cleaves the PCR products at nucleotides 588 of survivin cDNA and generated 2 smaller pieces (64 and 492 bp for survivin and 64 and 374 bp for survivin-ΔEx3).

Discussion

In the present study, we determined the expression pattern of survivin splice variants in patients diagnosed with bladder cancer. The main aim of the study was then to examine any potential correlation of gene expression with degree of malignancy, pathological behavior, and the recurrence of bladder cancer.

Our results revealed that survivin and/or survivin-ΔEx3 were expressed in 42.8% of cases in group 1 (primarily detected tumors) and 60% of cases in group 2 (recurrent tumors). In the original report by Swana and coworkers,[13] where expression of survivin was determined by immunohistochemistry, no detectable survivin was reported in normal transitional cells, but it was present in 78% of tumoral specimens. In contrast, Lehner and colleagues have reported that survivin is not only detectable in tumoral specimens, but also is present in some nontumoral specimens of the bladder.[14] Also recently, Gazzaniga and colleagues have reported that they were able to detect survivin expression by RT-PCR technique only in 9 out of 30 specimens (< 30%) of superficial bladder cancer.[15] Accordingly, in another report by Nakanishi and colleagues, survivin protein was detected by immunohistochemistry in only 12.7% of TCC specimens.[16]

The high expression of survivin in specimens 1 and 2 in group 1 as well as a moderate expression of the gene in specimen 2 of group 2 (which had a high mitotic activity and nucleus-plasma ratio) is in complete agreement with the role of survivin in regulating cell proliferation and its upregulation in the G2/M phase.[17] In other words, because of the increased rate of cell division in these specimens, the expression of survivin is high.

What differentiates specimen 3 in group 1 from other cases, despite having high mitotic activity and high nucleus-plasma ratio, was the presence of the cells with a big granulated nucleus, a characteristic of necrotic cells. There was also a significant amount of necrosis in specimens 3, 9, and 10 in group 2. Since there is a continuous spectrum of cell death by necrosis or apoptosis,[18] the lack of survivin gene expression in these cases is predictable.

Specimen 5 in group 2, which was survivin positive, had some degree of necrosis, and it was expected to be survivin negative. From a closer look at the pathological and microscopic report

and from the fact that the tumor was heterogeneous in nature, we speculated that the sampling might have been taken from parts of the tumor lacking obvious necrosis. What makes this case more complicated is a transformation of the tumor from TCC to SCC (which is more aggressive); since there is little information about the nature of this transformation, a reliable interpretation of the case is difficult.

Specimen 1 in group 2 was from a 76-year-old man being followed up for bladder cancer, and after a long period from primary tumor diagnosis, a small tumor of low stage with a negative urine cytology result had been found. All of these data suggest an appropriate biological behavior of the tumor, and the sole expression of survivin (but not survivin-ΔEx3-which has poorer prognosis) could have been anticipated. The absence of survivin-ΔEx3 variant in this case and in specimen 5 in group 1 makes them different from other survivin-positive cases.

Despite previous reports on the presence of survivin-ΔEx3 and survivin-2B in a variety of tumoral tissues and cell lines, there are no reports on the involvement of the same variants in bladder tumors. Mohatka and colleagues detected these alternatively spliced variants for the first time in renal cell carcinoma cell lines.[8] The variants differ from each other, not only because of having different sizes, but also because of their different antiapoptotic activities.[19]

Primers were BLAST compared against the human genome to make sure they do not have nonspecific complementary sequences on the genome. Also, we used nested RT-PCR to increase the specificity and sensitivity of the reaction. Thus, we can conclude that the 2 amplified bands are indeed different variants of survivin. However, we failed to detect one of the previously reported variants of survivin, survivin-2B, in the examined specimens. This might be due to the low number of tumors with low stages in the current study. A reduced expression of survivin-2B variant has been shown to be correlated with a poor prognosis of gastric carcinoma.[20] Also, it is claimed that expression of these variants might have a role in tumor progression and clinical behavior of soft tissue sarcomas,[21] colorectal carcinomas,[22] medulloblastoma,[23] breast cancer,[24] and other cancers. To examine this hypothesis, we reviewed the patients' archival records and found that patients who were positive for survivin-ΔEx3 had either been operated on by cystectomy or had recently been a candidate for cystectomy. In other words, the presence of the variant is correlated with a poor diagnosis and a more rapid disease recurrence.

Conclusion

In conclusion, it seems that under various physiological and pathological conditions, apoptosis regulation depends not only on the extent of survivin gene expression, but also on how its primary transcript is processed to produce different splice variants. Therefore, determining the generation of different variants of the gene in different tumors and normal tissues would provide valuable diagnostic and prognostic information.

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