

Evaluation of sensitivity and specificity of urine survivin as a new molecular marker in diagnosis of bladder tumors

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Abstract

Bladder cancer is one of the most common forms of cancers in the world. The current gold standards for its diagnosis are cystoscopy and urine cytology. Cystoscopy, a naked eye assessment of the bladder, is invasive, uncomfortable and costly with a great deal of personal variability in its results; while urine cytology has high specificity but low sensitivity, particularly for low-grade lesions. Therefore, there is a need for a molecular tumor marker assay capable of detecting bladder cancer with high sensitivity and specificity. A growing list of tumor markers in urine has been introduced so far, but neither of them has been able to replace the current diagnostic methods. Survivin, an inhibitor of apoptosis (IAP) capable of regulating both cell proliferation and apoptosis, has been recently defined as a universal tumor antigen and as the fourth most significant transcript expressed in human tumors. It has been reported to have 100% sensitivity and 95% specificity for detection of bladder cancer. In the present study, the sensitivity and specificity of survivin as a tumor marker in detecting new and recurrent cases of bladder cancer has been evaluated by nested RT-PCR technique. Our results revealed that survivin could be detected in most patients (11/13, sensitivity=0.84) as well as some healthy volunteers with no obvious sign of bladder cancer (6/13, specificity=0.53). Also, in this work, for the first time, the presence of two alternatively spliced variants of survivin (survivin-2B and survivin-ΔEx3) urine is being reported. Interestingly, the presence of survivin-ΔEx3 was better correlated with malignant lesions of bladder compared to the survivin

expression (sensitivity=0.84, specificity=0.92).

Keywords: Survivin; tumor marker; Bladder cancer; Urine, RT-PCR

INTRODUCTION

Bladder cancer is one of the most common forms of cancer worldwide. It is the fourth most common type of cancer in men and the eighth most common type in women. Incidence of bladder cancer increases with age, where people over the age of 70 develop the disease 2 to 3 times more often than those aged 55-69 and 15 to 20 times more often than those aged 30-54 (Landis *et al.*, 1998). Despite the fact that there is no comprehensive statistical report on the incidence rate of bladder cancer in Iran, but according to the clinical reports it has a very high incidence rate and most of the cases are in the form of transitional cell carcinoma (TCC), similar to the one in Europe and North America.

Tumor growth depends on two main factors, cell proliferation and cell death by apoptosis (Guo and Hay, 1999). Apoptosis is a form of cell death characterized by morphological, biological and genetic features. Abnormalities of apoptosis may lead to uncontrolled cellular proliferation and ultimately carcinogenesis. Several studies have reported significant correlations between apoptosis and prognosis in malignant tumors including lung cancer, breast cancer and esophageal cancer (LaCasse *et al.*, 1998).

Two protein families are involved in the control of apoptosis: BCL2 and IAP (Inhibitor of Apoptosis Protein). IAPs are a group of evolutionary conserved

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proteins characterized by the presence of one to three domains known as baculoviral IAP repeat (BIR) domains, which is necessary for the anti-apoptotic property of IAPs (Adams and Cory, 1998).

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 (O'Driscoll *et al.*, 2003). Survivin also plays critical roles in regulating the cell cycle and mitosis. Its primary expression in almost all human malignancies, and low or absent expression in most normal tissues, suggests that it would be a good diagnosis/prognosis marker as well as an ideal target for cancer-directed therapy (Velculescu *et al.*, 1999). Extensive studies have been carried out to elucidate the mechanism of its function, however, its role in regulating survival and cell cycle is poorly understood (Altieri and Marchisio, 1999).

Mahotka and colleagues (1999) cloned and characterized two novel splice variants of survivin, lacking exon 3 (survivin- Δ Ex3) 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. The same group also reported a conservation of antiapoptotic properties for survivin- Δ Ex3 and a markedly reduced antiapoptotic potential for survivin-2B. The reduced anti-apoptotic activity of survivin-2B is possibly due to a dominant-negative mechanism of competitive binding to the interactive partners of survivin (Islam *et al.*, 2000).

Considering the necessity of using molecular markers for diagnosis and determining the prognosis of bladder tumors and also because of the potential application of survivin as a specific tumor marker for cancers in general, we have evaluated the expression of survivin in the urine of bladder cancer patients and also healthy volunteers by means of RT-PCR. By determining the profile of expression for splice-variants of the gene, we aimed to determine the potential correlation between the presence of the variants and the malignant behavior of the tumor.

MATERIALS AND METHODS

Sample preparation: Urine samples were obtained during Dec 2002 to July 2003 either from patients' referred to the Labbafi-Nejad Medical Center or from healthy volunteer students of Tarbiat Modarres dormitories. Patients were examined either because of being suspected of having bladder tumor (primary or recurrent) or because of follow up procedure following

transurethral resection of bladder tumor by cystoscopy. We also used cystoscopy result as our gold standard test for patient group. All sampling were supervised by an urologist specialist and categorized into three sub-groups: 1) Patients suspicious of having bladder cancer (according to their clinical criteria such as hematuria). 2) Patients with previous history of bladder cancer whom were in their follow-up period of their tumor progression. 3) Persons whom are neither suspicious of having bladder cancer nor having a prior history of cancer (control group).

A total number of 26 samples were collected and for each sample 50 ml of urine were taken from each person either naturally or prior to cystoscopy. Samples were kept on ice and quickly transferred to the laboratory. To collect the shed bladder cells in urine, samples were centrifuged at 9000g for 10 min.

RNA extraction: Total RNA was isolated from urine cellular pellets using the RNX plus solution (Cinnagen, Iran) according to the manufacturer's instructions and as described before (Nikpoor *et al.*, 2004). The purity and integrity of the extracted RNA was evaluated by optical density measurements (260/280 nm ratios) and by visual observation of samples electrophoresed on agarose gels. Both methods confirmed the integrity of the extracted RNA with little or no protein contamination.

RT-PCR reaction: Specific primers of human β 2m (as an internal control; Accession number: NM-004048) and human survivin (Accession number: U75285) were designed by using Genrunner software (version 3.6). The sequences of the designed primers are as follow:

External, forward primer: 5'-TGGCAGCCCTTTCTCAAG-3' External, reverse primer: 5'-GAGAGAGAAGCAGCCAC-3'.

These primers amplified a 632 bp segment of human survivin cDNA located between nucleotides 77-708. Internal, forward primer: 5'-ACCACCGCATCTCTACATTC-3' Internal, reverse primer: 5'-CTGGTGCACATTTCAAGAC-3'.

These primers amplified a 556 bp segment from human survivin cDNA located between nucleotides 96-651. β 2m forward primer: 5'-TCG CGC TAC TCT CTC TTT CTG-3' β 2m reverse primers: 5'-GCT TAC ATG TCT CGA TCC CAC-3'.

These primers amplified a 334 bp segment from human β 2m cDNA located between nucleotides 41 to 374.

Complementary DNA (cDNA) synthesis reactions were performed using 5 μ g RNA and MMLV reverse

transcriptase (Gibco BRL, Germany) with oligo (dT)₁₈ priming in a 20 µl reaction as described elsewhere (Nikpoor *et al.*, 2004).

The designed primers as well as the oligo (dT)₁₈ primer were synthesized by MWG Company (Germany) as high purified salt free (HPSF) grade. All designed primers were blasted (NCBI, BLAST) with 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 (Sambrook and Russel, 2001). The PCR amplification was performed for 25-35 cycles. The cycling conditions were as follows: 94°C for 30 sec, 55°C for 30 sec, 72°C for 1 min and a final extension at 72°C for 10 min. PCR products were then separated on a 1.5% agarose gel and visualized by ethidium bromide staining.

To make sure of using equal amount of RNA in all reactions, we used β2m as an internal control. For each sample, the RT-PCR was performed under similar conditions, but in two separate tubes, for both survivin and β2m (except for the number of cycles).

Restriction enzyme digestion: To confirm the identity of PCR products of survivin variants, we have determined the restriction size pattern of all amplified products digested with *MboI* restriction enzyme (Fermentas). The enzyme cuts survivin cDNA at nucleotide 588 generating two shorter bands that is simply detectable on 8% poly-acrylamide gel electrophoresis.

Statistical analysis: Urine samples were classified as either positive or negative for survivin and survivin-ΔEx3 gene expression in both tumoral and non-tumoral groups. The statistical analysis of the correlation between expression of survivin/survivin-ΔEx3 and tumoral/non-tumoral state of the samples was performed using the two-tailed Pearson test on SPSS software for windows (version 11, Chicago).

RESULTS

RT-PCR optimization: To find the optimum number of amplification cycles, we first employed external primers 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). Due to the weak intensity of the signal, nested-PCR was performed on the product of the first round of PCR, using internal primers and

for 25-35 cycles. The result revealed two bands with sizes of 556 and 438 bp, as expected from the sizes have been 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 β2m and 35 cycles, first round, and 30 cycles, second round, for survivin.

Evaluating the expression of survivin and its splice-variants in urine samples: Overall, 26 urine specimens were collected. The age of the persons (patients and volunteers) was 17-82 years old. Details of personal and clinico-pathological characteristics of all cases are listed in Table 1. As expected, β2m was expressed in all specimens (Fig. 1). Nested RT-PCR results on the same samples revealed an expected band for survivin with a size of 556 bp; as well as another band with the approximate size of 438 bp (Fig. 1). In case U3-19, in addition to the 556 bp band, another band with an approximate size of 625 bp was observed (Fig. 1B).

The experiment was repeated at least twice for all samples, the re-appearance of the smaller band in the same samples points to the potential detection of a survivin splice-variant, which was previously reported in other cancers (Mahatka *et al.*, 1999). Changing the PCR conditions (i.e. increasing the annealing temperature and/or decreasing the concentrations of the primers) didn't affect the intensity of the bands (data not shown).

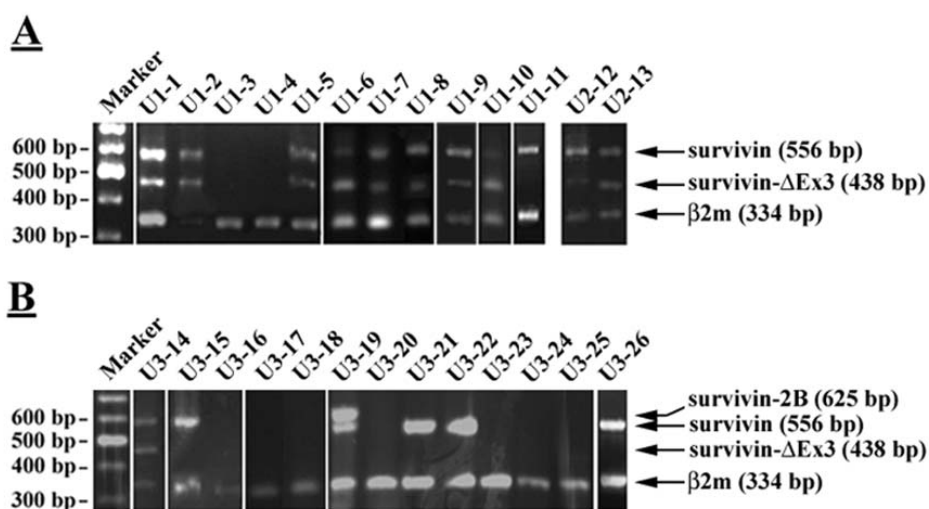
In group 1 (patients suspicious of having bladder cancer, which includes 11 cases), cases U1-3 and U1-4 showed no detectable signal for survivin, while U1-11 showed only the 556 bp band, and the rest of the cases showed two bands with the sizes of 438 and 556 bp (Fig. 1. cases U1-1 to U1-11). Both cases in group 2 (cases with previous diagnosis of bladder tumors and currently under follow-up surveillance) showed both 438 and 556 bp bands (Fig. 1A). In control group (cases U3-14 to U3-26), 4 cases (U3-15, U3-21, U3-22 and U3-26) showed only the 556 bp band, one case (U3-14) showed both 556 and 438 bp bands, and one case (U3-19) had two bands with 556 and 625 bp bands (Fig. 1B).

In the patients groups, the sensitivity of the urine survivin and survivin-ΔEx3 test new or recurrent bladder cancer detection was 0.84 and the specificity was 0.53 and 0.92, respectively.

Confirming the identity of survivin variants by means of enzymatic digestion: The identity of the amplified bands in PCR, i.e. the bands corresponding to the survivin splice-variants, was confirmed by

Table 1. A brief description of persons, whom their urine samples were used in the study.

Case	Age (Year)	Sex	Reason for taking sample	Smoking
U1-1	47	♂	Macroscopic hematuria	+
U1-2	68	♂	Macroscopic hematuria	+
U1-3	67	♂	Macroscopic hematuria	-
U1-4	72	♂	Macroscopic hematuria	+
U1-5	66	♂	Macroscopic hematuria	+
U1-6	59	♂	Macroscopic hematuria	-
U1-7	41	♂	Macroscopic hematuria	+
U1-8	82	♂	Macroscopic hematuria	Not available
U1-9	56	♂	Macroscopic hematuria	+
U1-10	68	♂	Macroscopic hematuria	+
U1-11	60	♂	Macroscopic hematuria	Not available
U2-12	17	♂	Painful urination	-
U2-13	46	♂	Macroscopic hematuria	-
U3-14	67	♂	High PSA/Bladder outlet difficulty	-
U3-15	56	♂	Bladder outlet obstruction	+
U3-16	46	♂	Bladder outlet obstruction	+
U3-17	47	♂	Double J removal	-
U3-18	41	♂	Double J removal	-
U3-19	27	♂	volunteer	+
U3-20	24	♂	volunteer	-
U3-21	25	♂	volunteer	-
U3-22	27	♂	volunteer	-
U3-23	26	♂	volunteer	-
U3-24	27	♂	volunteer	-
U3-25	27	♂	volunteer	-
U3-26	20	♂	Bladder outlet difficulty	-

**Figure 1.** RT-PCR analysis of the expression of β 2m and survivin in urine samples of: A) Patients suspicious of having bladder cancer (cases U1-1 to U1-11) as well as patients with recurrence of bladder cancer (samples U2-12 and U2-13). B) Control group with no clinical signs of bladder disease (U3-14 to U3-26).

means of restriction enzyme digestion. For this purpose, we chose *MboI* enzyme, which cuts survivin cDNA at nucleotide 588 to generate two shorter segments (64 and 492 bp and 64 and 374 bp) for survivin and survivin- Δ Ex3, respectively. The enzyme also generates three shorter segments (64, 235 and 326 bp) for survivin-2B (Fig. 2B). The later results confirmed the identity of amplified products as survivin and its splice variant, survivin- Δ Ex3.

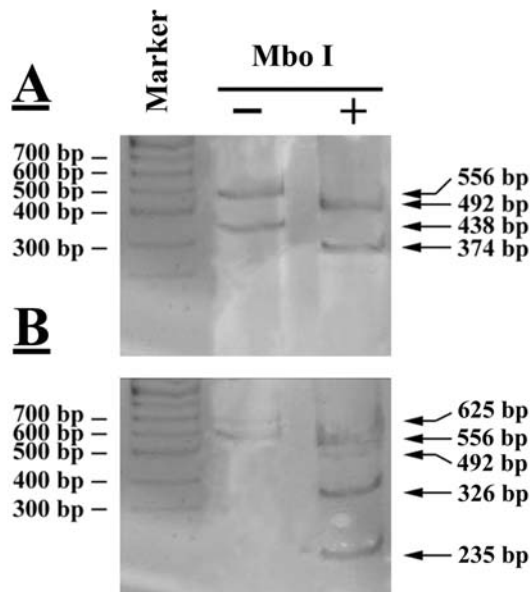


Figure 2. Electrophoresis of digested products of amplified survivin segments by *MboI* on 8% poly acrylamide gel. The enzyme cleaves the PCR products at nucleotides 588 of survivin cDNA and generated two smaller pieces (64 and 492 bp) for survivin and (64 and 374 bp) for survivin- Δ Ex3, and 3 segments (64, 235 and 326 bp) for survivin-2B.

Correlation between survivin/survivin- Δ Ex3 expression and tumoral/non-tumoral state of samples: Statistical results revealed a significant strong correlation of 0.701 between the expression of survivin- Δ Ex3 and the disease state of the samples ($P < 0.001$). Similarly, a significant moderate correlation of 0.401 has been found for survivin ($P < 0.04$).

DISCUSSION

In the present study, we have tested the presence of survivin splice-variants in the urine of patients suspicious of having bladder cancer as well as healthy volunteers. The main aim of the study was then to evaluate the diagnostic value of urine survivin gene expres-

sion to predict bladder cancer. Our results revealed that:

1- The expression of survivin was detectable in most patients suspicious of having bladder cancer (9 out of 11), both patients with recurrent bladder cancer and some of healthy volunteers (6 out of 13). The obtained data suggest that the specificity and sensitivity of Survivin mRNA in urine is clearly less than the original report. In the original report by Smith *et al.* (2001), where the presence of survivin protein and mRNA in the urine of bladder cancer patients was determined by Western blotting and nested RT-PCR, a sensitivity of 95% and specificity of 100% for survivin was reported. Also, according to Swana and colleagues (1999) there was no detectable survivin in normal transitional cells, but the protein was present in 78% of tumoral samples. In contrast, Lehner and colleagues (2002) reported that survivin is not only detectable in tumoral samples, but also is present in some non-tumoral samples of bladder. Also, recently Gazzaniga and colleagues (2003) were able to detect survivin expression by RT-PCR technique in just 9 out of 30 samples (<30% of cases) of superficial bladder cancer. Accordingly, in another report by Nakanishi *et al.* (2002) survivin protein was detected by immunohistochemistry in only 12.7% of TCC samples. Therefore, according to our results, the sensitivity and specificity of survivin as a bladder tumor marker is remarkably less than it is originally claimed (0.84 and 0.53 respectively).

2- Our results also demonstrated, for the first time, the presence of survivin spliced-variants (survivin- Δ Ex3 and survivin-2B) in bladder tumors. Despite the previous reports on the presence of survivin- Δ Ex3 and survivin-2B in a variety of tumoral tissues and cell lines, there was no report on the involvement of the same variants in bladder tumors. Mohatka and colleagues (1999) detected these alternatively spliced-variants for the first time in RCC cell line. The variants differ from each other not only because of having different sizes, but also because of their different anti-apoptotic activity.

Since we had blasted the designed primers against the human genome to make sure they don't have non-specific complementary sequences on the genome and also because we used nested RT-PCR to increase the specificity and sensitivity of the reaction, we concluded that the two amplified bands are probably different variants of survivin. To confirm our conclusion, the amplified products were subjected to enzymatic digestion, which generated smaller segments with predicted sizes for survivin variants. These variants have different anti-apoptotic property and their ratio is probably

regulated by complex mechanisms. A reduced expression of survivin-2B variant has shown to be correlated with a poor prognosis of gastric carcinoma (Krieg *et al.*, 2002). Also, it is claimed that the expression of these variants might have a role in tumor progression and clinical behaviour of soft-tissue sarcomas (Taubert *et al.*, 2005), colorectal carcinomas (Suga *et al.*, 2005), medulloblastoma (Fangusaro *et al.*, 2005), breast cancer (Ryan *et al.*, 2005) and other cancers. To examine the hypothesis for the present study, we reviewed the patients' archival records and found that patients whom were positive for survivin- Δ Ex3 had been either operated by cystectomy or been candidate for radical cystectomy in a short period of time. In other words, the presence of the variant is probably correlated with poor prognosis and more rapid recurrence of the disease. However, the later conclusion needs to be further examined on archival collection of formalin-fixed paraffin-embedded samples of bladder tumors.

3- Survivin-2B variant was only detected in one case in control group (U3-19). The restricted expression of the variant in the control group is in agreement with the previous works, which reported a good prognosis for this variant (Krieg *et al.*, 2002). In survivin-2B, part of intron 2 (69 bp/23 amino acids) is retained in the processed transcript as a cryptic exon (exon-2B). This led to a change in the structure of BIR domain and consequently a decrease in the anti-apoptotic potential of the protein. It is also hypothesized that the reduced activity of survivin-2B is probably due to its competitive binding to the interactive partners of survivin (Islam *et al.*, 2000).

4- We report here for the first time, a direct and strong correlation between survivin- Δ Ex3 and bladder cancer (0.701 and $P < 0.001$). The variant was detected in the urines of 11 out of 13 patients. Interestingly, in 2 cases that we failed to detect the variant, there was also no detectable expression for survivin. Therefore, the sensitivity of the variant (0.84) is similar to that of survivin in detecting bladder tumors. On the other hand, the expression of survivin- Δ Ex3 was detected in only one case of control group (false-positive), which is in sharp contrast to the rate of false-positives for survivin (7 cases). Therefore, the specificity of survivin- Δ Ex3 variant in differentiating between bladder tumors and other diseases of the organ is significantly higher than the one for survivin (0.92 and 0.53 respectively).

In conclusion, it seems that under various physiological and pathological conditions apoptosis regulation is 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 information.

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