Indian Journal of Medical Sciences
(INCORPORATING THE MEDICAL BULLETIN)
VOLUME 59 JUNE 2005 NUMBER 6
ORIGINAL CONTRIBUTIONS

SURVIVIN PROTEIN EXPRESSION POSITIVELY CORRELATED WITH PROLIFERATIVE ACTIVITY OF CANCER CELLS IN BLADDER CANCER

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ABSTRACT

CONTEXT: *Survivin is an inhibitor of apoptosis that is selectively over-expressed in common human cancers, but not in normal tissues, and that correlates with aggressive disease and unfavorable outcomes.* **AIMS:** *To identify the role of survivin in bladder carcinogenesis and the correlation between survivin protein expression and the occurrence of spontaneous apoptosis, proliferative activity of cancer cells.* **SETTINGS AND DESIGN:** *Retrospective analysis.* **METHODS AND MATERIAL:** *Bladder transitional cell cancer (BTCC) tissue samples for 128 patients were investigated, with normal bladder tissues serving as controls. From these tumor samples, 42(32.8%) were grade I, 59(46.1%) were grade II, and 27(21.1%) were grade III; 72(56.2%) were superficial, 56(43.8%) were invasive. The survivin protein level was quantified by Western blot analysis. The apoptotic index (AI) using in situ labeling apoptotic DNA fragment kit and the Ki-67 labeling index (Ki-67LI) with an anti-Ki-67 monoclonal antibody were analyzed in these tumors, respectively.* **STATISTICAL ANALYSIS USED:** *Differences in the S/β ratio between tumor grade and stage were evaluated by using unpaired t-test and F-test. The relationships between the S/β ratios and AIs, Ki-67LIs of tumors were evaluated by Pearson correlation coefficient.* **RESULTS:** *High survivin levels were detected by Western blot analysis in tumor tissue extracts. None of the expression of survivin protein was found in normal bladder tissues. Survivin levels were significantly different from different clinical stages and pathological grades of the tumors ($P < 0.01$, respectively). Pearman correlation coefficient test revealed a positively correlation between survivin protein level and the proliferative activity ($P < 0.001$) and failed to find significant correlation between AI and survivin protein level ($P > 0.05$).* **CONCLUSIONS:** *Survivin protein expression played an important role in the malignant progression of BTCC.*

KEY WORDS: *Apoptosis, bladder cancer, proliferative activity, survivin*

INTRODUCTION

It has been claimed that bladder cancer represent a disease with variable clinical behavior, which shows to a clear tendency to early relapse in almost 60% of patients, independent of clinical prognostic variables.[1] The high rate of recurrence poses challenges for appropriate follow-up diagnosis and treatment. 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.

Survivin, a novel human number of the inhibitor of apoptosis protein (IAP) family, has been identified recently. It has been suggested to directly inhibit caspase-3 and –7 activity or conjugate caspase-9, and regulate the G_2/M phase by interact with spindle microtubules.[2],[3] Survivin is expressed in fetal development, in normal endometrium primarily during the secretory phase of the menstrual cycle and in cancers, whereas no transcripts were detected in terminally differentiated adult tissues. Expression of survivin on breast, neuroblastoma, lung, esophageal, and colorectal cancers correlates with an unfavorable prognosis, which is shortened survival and/or a shortened time to recurrence,[4]– [7] 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.

Bladder cancer is one of the malignant tumors which even in the early stage, many patients develop cancer recurrences. If the survivin gene expression is a useful marker for the malignant potential of bladder cancer, therapeutic methods could be changed according to the expression levels of the survivin gene. However, only a few studies have dealt with survivin expression in bladder transitional cell carcinoma (BTCC) with a small number of patients, and the results were controversial.[8],[9] Further, the correlations between survivin gene expression and other biological factors for bladder cancer remain unclear. In the present study, we examined the expression of survivin by Western blot in a large serial of patients with BTCC to evaluate the role of survivin in bladder carcinogenesis and the relationships between survivin protein expression and biological factors such as the occurrence of apoptosis, proliferative activity of cancer cells.

SUBJECTS AND METHODS

Patients and tissues

From January to June 2004, we retrospectively analyzed survivin protein expression and its relationship with the clinicopathological parameters, apoptosis, and proliferative activity of cancer cells in 128 cases of bladder cancer patient (78 men and 50 women; mean age at time of surgery, 66.2 years; median, 68; range, 44–86), on whom surgical resection of the primary BTCC had been performed between

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2001 and 2003 in our hospital. Operations consist of total cystectomy, partial cystectomy, and transurethra resection of bladder tumor (TURBT). None of the patients received preoperative chemotherapy or radiation therapy. Informed consent was obtained from all patients under study. Fresh specimens were recovered immediately after resection and half of each tissue section representative of the tumor was immediately frozen in liquid nitrogen within 10 min of excision and stored at –80°C until use, and the other half of the tissue was fixed in 10% buffered formalin and embedded in paraffin. Standard 4 mm thick tissue sections were stained with haematoxylin and eosin (H&E) and examined by light microscopy. In all tumor specimens, the amount of tumor cells was equal to or exceeded 80% of the sample, confirmed by histological examination. Tumor grading and staging were performed according to the principles outlined by the WHO and the TNM" UICC. Of the tumor samples, 42(32.8%) were grade I, 59(46.1%) were grade II, and 27(21.1%) were grade III; 72(56.2%) were superficial, 56(43.8%) were invasive. We obtained tumor and noncancerous bladder epithelium samples, distant enough from the tumors from the 128 patients.

Protein extraction and Western blot analysis

Tissue extracts were prepared from frozen tissues by a standard extraction protocol.[10] An amount of 30 µg of total protein from each tissue extract was separated on a 12% gradient polyacrylamide/SDS minigels (Bio-Rad Mini Protean II). After transfer by elecroelution to nitrocellulose membranes, blots were blocked with 5% milk in 80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 100 mM NaCl, 0.1% Tween 20, pH 7.5 for 1 h and incubated with rabbit antihuman survivin Ab(dilution1:1000, Clone AF886; R&D systems, Wiesbaden, Germany) overnight at 4°C. After washing, the membranes were incubated with horseradish perixidase-conjugated secondary antibody (diluted 1:2000, DAKO, Glostrup, Denmark) for 1 h at room temperature. Bands were visualized using the exhanced chemiluminescence system (ECL, Amersham International). Controls were made with an exchange of the primary antibody for an antibody preabsorbed with immunizing peptide (100 ng/40 ng IgG). For normalization, the band intensity of survivin protein was related to that of β-actin, which was run in parallel blots, and the survivin: β-actin ratios (S/β) were calculated.

Detection of apoptotic cells in paraffin sections

The slides of paraffin-embedded tumor samples were dewaxed in xylene and ethanols in descending concentrations, and washed in double-distilled water (DDW) two times for 5 min. Subsequently incubation with proteinase K solution containing 400 µl proteinase stock solution (25 mg proteinase K in 2.5 ml DDW) in 200 ml Tris–HCl buffer (pH 7.0) was performed for 15 min at room temperature. After four washes in DDW for 2 min the sections were incubated with terminal transferase mixture (Boehringer Mannheim, Germany) under a cover slip for 1 h at 37°C.

After three washes in TB buffer (300 mM sodium chloride and 30 mM sodium citrate in 1000 ml DDW, pH 7.5) for 5 min and one wash

in buflfer 1 (100 mM Tris–HCl and 150 mM NaCl in 2000 ml DDW, pH 7.2) for 5 min, the sections were immersed in buffer 2 for 30 min. For preparation of this buffer 0.1 g of blocking reagent 0.5% was mixed for 1 h at 60°C with 200 ml of bufier 1. Subsequently, two washes in buffer 1 for 5 min were followed by incubation with alkaline phosphatase-conjugated Fab fragments of polyclonal sheep antidigoxigenin antibody at a dilution of 1:2000.

Three washes in buffer 1 for 5 min and one 5 min wash in a buffer containing 100 mM Tris–HCl, 100 mM NaCl, and 50 mM $MgCl_2$ in 1000 ml DDW, pH 9.5 (buffer 3) were then followed by incubation with nitroblue tetrazolium (NBT)solution, first for 30 min at room temperature in the dark and then overnight at 4°C in the dark. For preparation of NBT solution, 22.5 µl NBT stock solution containing 1 g NBT in 9.33 ml 70% dimethylformamide solution was mixed with 17.5 µl bromochloroindolylphosphate, 5mM levamisole, and 5000ul buffer 3. On the next day after a wash in running tap water for 10 min the slides were counterstained in nuclear fast red for 10 min. After two additional washes in DDW, the sections were mounted with Aquatex.

Since the enzymatic reaction described labels both apoptotic cells and areas of necrosis, only those labeled cells were regarded as positive that showed additional characteristics of apoptosis, e.g., isolated localization within an intact cell complex without an inflammatory reaction. between 1000 and 2000 cancer cells/ case were examined by an observer (Wen XG). The results were expressed as apoptotic index (AI, percentage of immunostained apoptotic cancer cells).

Ki-67 immunohistochemistry

Tissue sections were deparaffinized in two five-minute changes of xylene and were rehydrated through alcohols to distilled water. Endogenous peroxidase activity was blocked with 1% H_20_2 in methanol for 10 min. Subsequently, sections were subjected to antigen retrieval by heating in a microwave oven in citrate buffer (pH 6) for a total of 10 min (i.e., two 5-minute periods with replacement of evaporated buffer in between). Following microwave antigen retrieval, the primary murine monoclonal antibody to the Ki-67 protein (MIB-1, Immunotech, Cedex, France) was applied overnight at a 1:50 dilution at 4°C. The slides were then sequentially incubated with biotinylated horse antimouse immunoglobulin (Vector Laboratories, Burlingame, CA; 1:500 for 30 min) and streptavidin-horseradish peroxidase (Zymed Laboratory Inc., San Francisco, CA; 1:200, for 30 min). 3-3' Diaminobenzidine (DAB) (Sigma Chemicals, St. Louis, MO) was used as the chromagen. Sections were lightly counterstained with hematoxylin. To obtain the Ki-67 labelling index (Ki-67LI, percentage of immunostained cancer cells), between 1000 and 2000 cancer cells were examined using an observer (Wen XG).

Statistical analysis

Differences in the S/β ratio between tumor grade and stage were evaluated by using unpaired *t*-test and *F*-test. The relationships between the S/β ratios and AIs, Ki-67LIs of tumors were evaluated by Pearson correlation

coefficient.

RESULTS

Correlation between S/β ratio and clinical stage, pathologic grade of BTCC

By Western blotting, the survivin protein was detected in 98/128 (76.6%) tumor samples, but not in normal tissues [Figure 1]. *S/β* ratio of tumor samples range from 0 to 1.203(mean±*s*, 0.328 ± 0.335). As shown in Table 1, *S/β* ratios were significantly different with different clinical stages (t = 4.164, P < 0.001) and pathological grades (F = 9.557, P < 0.001).

Correlation between S/β ratio and AI, Ki-67LI

Figure 1: Western blot of survivin and β-actin in tumor (1–8) and normal tissue (9–11) extracts, positive control (12). Up line: survivin; low line: β-actin

Apoptotic cells and Ki-67 expression in tumor section were shown in Figures 2 and 3, respectively. The mean values of AI, Ki-67LI of 128 tumors were as follows: AI, 2.1% (range: 0.3–25.4%, median:1.2%); Ki-67LI, 22.8% (range: 0–61.9%, median: 17.9%). We evaluated the correlations between tumor *S/β* ratios and these biological factors by the

Table 1: Correlation between *S/β* ratios and clinical stage, pathologic grade

	No.	S/β *ratio*	P *value*
Clinical stage			
Tis–T_1	72	0.225 ± 0.259	<0.001
T_2–T_4	56	0.459 ± 0.376	
Pathologic grade			
I	42	0.209 ± 0.273	<0.001
II	59	0.313 ± 0.265	
III	27	0.546 ± 0.448	

S/β ratio: survivin/β-actin ratio.

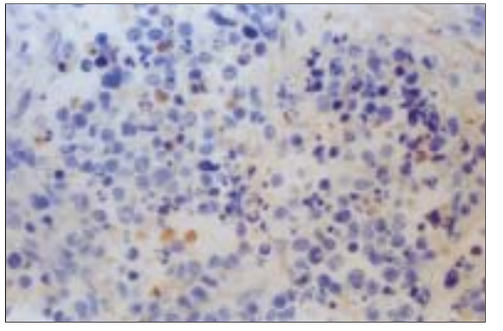

Figure 2: Apoptotic nuclei labeled enzymatically by terminal transferase reaction in a pT3,G3 bladder transitional cell cancer

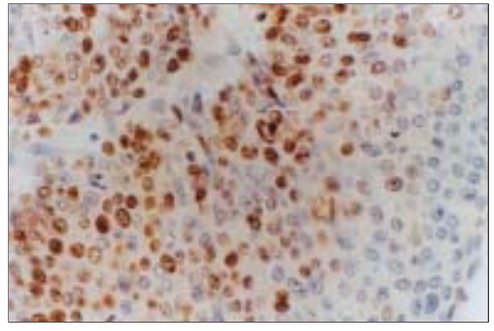

Figure 3: Ki-67 expression in a pT1, G2 bladder transitional cell cancer, with positive nuclear staining

Pearson correlation coefficient test, and found significant positive correlations between tumor *S/β* ratios and Ki-67LI (r_s = 0.836, P < 0.001), while tumor *S/β* ratios did not correlate with Ais (r_s = 0.121, P = 0.174) in BTCC. Figure 4 shows a significant positive correlation between tumor *S/β* ratios and Ki-67LIs in 128 BTCCs.

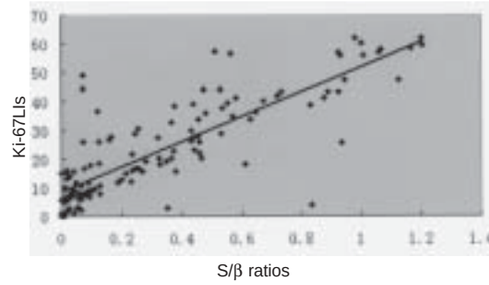

Figure 4: A significant positive correlation between tumor S/β ratios and Ki-67LIs in 128 BTCCs

DISCUSSION

In the present study, we evaluated by the presence of survivin expression in normal and cancerous bladder tissues. In normal tissues, no detectable levels of survivin protein expression were detected. Among tumor tissues, 98/128(76.6%) showed expression of survivin protein. When normalized with *β*-actin, the *S/β* ratios correlated well with clinical stage and pathological grade. This results consistent with the results of breast, lung, esophageal, and colorectal cancers that survivin expression is associated with both unfavorable histology and higher stage of disease.[4]–[7] In the studies of bladder cancer, the results were controversial.[8]–[9] Gazzaniga et al.[8] using RT-PCR to detect mRNA expression in 30 patients affected by primary superficial transitional cell carcinoma of the bladder, they found survivin mRNA expression did not correlated to clinical stage or multicentricity of the tumors. In this study, only a small number of patient were investigated and it is difficult to estimate the real amount of the gene expression level by traditional RT-PCR used in this study. It is reported that survivin had different mRNA splice variants and not all the variants translated to functional survivin protein.[11] In another study, Swana et al.,[9] who used immunohistochemistry to examine 36 cases of TCC of the urinary bladder, reported detecting survivin expression in 78% of 36 cases and even higher proportion of high-grade tumors (15 of 16 grades II and III tumors). Furthermore, they found that the time to first recurrence was significantly shorter in patients with survivin positive grade-I tumors than in those with survivin negative grade-I tumors. This results parallel with our study. It is reported that intravesical treatment of transitional cell carcinoma with Bacillus Calmette-Guerin and mitomycin C could affect the urinary survivin levels and patient outcomes.[12] In our study, all the patients with primary BTCC, which no chemotherapy or radiotherapy was used before surgery.

We also found that survivin expression significantly correlated with Ki-67LI, but not with AI. The survivin protein is reported to bind specifically to caspase-3 and –7 and to inhibit apoptosis in vitro.[2] Giodini et al.[3] reported that survivin expresses during the G_2/M phase of the cell cycle and that the disruption of survivin-microtubule interactions results in increased capase-3 activity and accelerated apoptotic cell death. Further, Ito et al.[13] reported that hepatocellular carcinoma cell

lines transfected with survivin show a significant decrease in cells in the G_0/G_1 phase and an increase in cells in the S and G_2/M phases. These findings indicate that the expression of the survivin protein may correlate not only with reduced apoptotic cell death but also with an increased proliferative activity of cancer cells. However, the antiapoptotic effect of survivin has recently been reported to be weaker than bcl-2 or xiap.[14] Thus, in bladder cancer, survivin gene expression may control cell proliferation rather than apoptosis.

BTCC is the most common cancer in urology system. Recurrences of bladder cancer occur in up to 60% of patients and constitute a formidable obstacle to long-lasting remissions, frequently anticipating muscle invasion, and disseminated disease.[1] Previously, the main predictors of outcome for these patients were the pathologic grade and clinicl stage of the tumor. Although these observations are extremely important, they do not consider the biology of the tumor, and thus, the behavior of a specific tumor may be disparate with its pathologic findings. In this study, we found survivin protein expression was associated with BTCC in late stage with poor cell differentiation and correlated well with the tumor proliferative activity. Survivin expression may play an important role in the malignant progression of BTCC through regulation tumor cell of proliferation. Using the molecular marker described in this study may help predict the clinical course and provide the biological character, and also survivin has great potential as a therapeutic target in BTCC.

ACKNOWLEDGEMENT

We thank all the patients for their participation in the study. We also thank Drs Xuepei Zhang, Zhiyong Wang, Jianwei Hao, and Jianguang Gao for devoting their time for sample collection and their practical perspective of patient care.

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