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. 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 is a novel human member 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. Survivin is expressed in fetal development, in secretory phase of the menstrual cycle and in cancers, whereas no transcripts were detected 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 aggressive behavior, which shows to a clear tendency to early relapse in almost 60% of patients, independent of clinical prognostic variables.

Survivin protein expression played an important role in the malignant progression of BTCC.
tumors were evaluated by Pearson correlation ratios and AIs, Ki-67LIs of
β blots were blocked with 5% milk in 80 mM S/
grade and stage were evaluated by using unpaired
test and t
tubulin, which
amount of 30
µ g of total protein from each
tissues by a standard extraction protocol.
Protein extraction and Western blot analysis
Tissue extracts were prepared from frozen
tissues by a standard extraction protocol. An amount of 30 µg of total protein from each tissue extract was separated on a 12% gradient polyacrylamide/SDS minigels (Bio-Rad Mini Protein II). After transfer by elecroelution to nitrocellulose membranes, blots were blocked with 5% milk in 80 mM NaHPO₄, 20 mM NaH₂PO₄, 100 mM NaCl, 0.1% Tween 20, pH 7.5 for 1 h and incubated with rabbit antihuman survivin Ab(dilution 1:1000, Clone AF886; R&D systems, Wiesbaden, Germany) overnight at 4°C. After washing, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody (diluted 1:2000, DAKO, Glostrup, Denmark) for 1 h at room temperature. Bands were visualized using the enhanced 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 buffer 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 buffer 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 MgCl₂ 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% H₂O₂ 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.d., 0.328±0.335). As shown in Table 1, S/β ratios were significantly different with different clinical stages (F = 4.164, P < 0.001) and pathological grades (F = 9.557, P < 0.001).

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

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

<table>
<thead>
<tr>
<th>Clinical stage</th>
<th>No.</th>
<th>S/β ratio</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tis–T1</td>
<td>72</td>
<td>0.225 ± 0.259</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>T1–T2</td>
<td>56</td>
<td>0.459 ± 0.376</td>
<td></td>
</tr>
<tr>
<td>Pathologic grade</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>42</td>
<td>0.209 ± 0.273</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>II</td>
<td>59</td>
<td>0.313 ± 0.265</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>27</td>
<td>0.546 ± 0.448</td>
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S/β ratio: survivin/β-actin ratio.

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.6–9 In the studies of bladder cancer, the results were controversial.10–13 Gazzaniga et al.10 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.13 In another study, Swana et al.9 who used immunohistochemistry to examine 36 cases of
lines transfected with survivin show a significant decrease in cells in the G1/G0 phase and an increase in cells in the S and G2/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. 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. Previously, the main predictors of outcome for these patients were the pathologic grade and clinical 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.

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REFERENCES