Immunohistochemical analysis of bcl-2 expression in transitional cell carcinoma of the bladder

H Shina, M Igawa, S Urakami, S Honda, H Shirakawa, T Ishitue

Abstract

Aims—To evaluate the expression of bcl-2 in transitional cell carcinoma (TCC) of the bladder; to compare bcl-2 expression with clinicopathological findings, p53 immunoreactivity, proliferating cell nuclear antigen (PCNA) expression, 2c deviation index (2cDI), 5c exceeding rate (5cER), and the mean nuclear area (MNA).

Methods—Cystectomy specimens from 77 patients with untreated, non-metastatic TCC of the bladder were studied. Expression of bcl-2, p53 and PCNA was detected immunohistochemically using the following monoclonal antibodies: bcl-2/124, DO-7 and PC10, respectively. Nuclear DNA content was analysed using static cytometry.

Results—Bcl-2 was expressed in 19 (24.7%) of 77 TCCs and in 74 (96.1%) of 77 normal samples of transitional epithelium (taken from normal tissue adjacent to the tumour in each case). In all cases, bcl-2 immunoreactivity was more intense in normal transitional epithelium than in TCC. In normal transitional epithelium and superficial TCC bcl-2 immunoreactivity was observed at the basal layer, and not at the invasive front. Bcl-2 immunoreactivity was inversely correlated with histological grade and p53 immunoreactivity, and was not correlated with the pT category, disease progression, PCNA expression, 2cDI, 5cER, and the MNA. No significant correlation was found between bcl-2 expression and overall survival.

Conclusions—Bcl-2 expression in TCC of the bladder seems to be associated with a less aggressive phenotype and does not play an important role in tumour progression.

(Keywords: Bcl-2, p53, PCNA, immunohistochemistry, bladder cancer, DNA content.)

The B cell leukaemia/lymphoma 2 gene (bcl-2) product is an inner mitochondrial membrane protein that inhibits programmed cell death (apoptosis), thus bestowing a survival advantage on cells expressing this oncprotein. Bcl-2 positive cells may then undergo a secondary event, rendering them susceptible to malignant transformation. The bcl-2 gene product has been intensively investigated in haematological malignancies, where overproduction of both bcl-2 mRNA and protein has been associated with a 14;18 chromosomal translocation. Recently, bcl-2 expression has been reported in several non-haematopoietic tumours, such as small cell and non-small cell carcinomas of the lung, melanocytic tumours, suggesting that the 14;18 translocation is not always required for the production of an immunohistochemically detectable bcl-2 protein. Bcl-2 immunoreactivity also has been demonstrated in glandular epithelium of hormone and growth factor regulating tissues, such as prostate, breast and thyroid, as well as in epithelium containing long-lived stem cells, such as the intestine. As there have been no reports regarding bcl-2 immunoreactivity in transitional cell carcinoma (TCC) of the bladder, the functional role of bcl-2 in this tumour remains unclear.

The p53 protein is a nuclear phosphoprotein regulating cell growth at the G1/S checkpoint. It is a tumour suppressor gene and plays an important role in eliminating cells with damaged DNA by inducing apoptosis. p53 gene mutations are the most common genetic alterations in human cancers, leading to the inhibition of DNA surveillance and repair and thereby increasing the risk of other gene mutations and chromosomal rearrangements. Thus, p53 gene mutations are associated with carcinogenesis and tumour progression.

The aim of this study was to elucidate the functional role of bcl-2 in TCC of the bladder using archival paraffin wax embedded tissue specimens and to compare bcl-2 expression with clinicopathological findings, p53 immunoreactivity, expression of the proliferating cell nuclear antigen (PCNA) and DNA parameters such as the 2c deviation index (2cDI) and the 5c exceeding rate (5cER) and the mean nuclear area (MNA).

Methods

Radical cystectomy specimens from 77 patients (19 women and 58 men) with untreated, non-metastatic TCC of the bladder were studied. The mean age of the patients at surgery was 62.3 years (range 37–81 years). Mean follow up was 45.3 months (range 19–135 months). Tumours were staged according to the UICC system. Disease progression was defined as an increase in the N or M, or both, categories during follow up. Patients who died of disease other than bladder cancer were excluded from the study. After surgery, patients with TCC of high histological grade (more than grade II) or...
high histological stage (more than pT2), or both, were treated with adjuvant chemotherapy comprising cisplatin, cyclophosphamide and adriamycin.

Tumour tissue and adjacent normal tissue specimens were fixed in 10% buffered formalin (pH 7.0) for 24–30 hours and embedded in paraffin wax. Five consecutive sections, 5 μm thick, were cut from each block and mounted on poly-l-lysine coated slides. One of the five sections was stained with haematoxylin and cosin for histological evaluation, was independently reviewed by two pathologists and graded as recommended by the World Health Organisation (WHO). The pT category of each TCC was assigned by examination of two or three representative sections in each case, as pTa, pT1, pT2, and >pT2. The histological findings are summarised in table 1. Multicentric tumour formation was observed in the bladders of all seven patients with grade I superficial TCC.

### Table 1  Histological grade and stage

<table>
<thead>
<tr>
<th></th>
<th>Grade I</th>
<th>Grade II</th>
<th>Grade III</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>pTa</td>
<td>4</td>
<td>9</td>
<td>0</td>
<td>13</td>
</tr>
<tr>
<td>pT1</td>
<td>3</td>
<td>15</td>
<td>3</td>
<td>21</td>
</tr>
<tr>
<td>&gt;pT2</td>
<td>1</td>
<td>16</td>
<td>26</td>
<td>43</td>
</tr>
<tr>
<td>Total</td>
<td>8</td>
<td>40</td>
<td>29</td>
<td>77</td>
</tr>
</tbody>
</table>

Bcl-2 IMMUNOSTAINING
Sections were deparaffinised routinely and were pretreated in a microwave at 700 W in 10 mM citrate buffer (pH 6.0) for five minutes. They were then allowed to cool for five minutes, re-microwaved and placed slowly in distilled water. Endogenous peroxidase activity was blocked by incubating the slides in 3% hydrogen peroxide for 30 minutes. The slides were washed in 0.05 M Tris buffer (pH 7.6) and incubated in 2% bovine serum albumin for 10 minutes and were then incubated for 12 hours at room temperature with a monoclonal antibody directed against bcl-2 (bcl-2, clone 124; Dako, Carpinteria, California, USA), diluted 1 in 50 in 0.05 M Tris buffer. After which, the slides were washed three times in Tris buffer and bcl-2 immunostaining was detected using the LSAB kit, as recommended by the manufacturer (Dako) with diaminobenzidine as the chromogen. The sections were counterstained with 0.5% methyl green. As a negative control, specific primary monoclonal antibody was replaced by the immunoglobulin fraction of non-immune mouse serum. Normal lymphocytes served as a positive control.

PCNA AND P53 IMMUNOSTAINING
Expression of p53 was detected immunohistochemically using a mouse anti-p53 monoclonal antibody (DO-7) (Dako) as described for bcl-2. Sections obtained from a patient with pT3b GIII exhibiting intense p53 positive staining served as a positive control. PCNA expression was detected in routinely processed, rehydrated sections by using a mouse monoclonal anti-PCNA antibody (PC10) (Dako). Non-immune mouse IgG replaced specific primary antibody in the negative control. Normal human tonsil served as a positive control.

EVALUATION OF IMMUNOSTAINING
Bcl-2 immunostaining was scored as the percentage of cells exhibiting definite immunoreactivity in at least 2000 neoplastic cells encountered in 20 randomly selected high power fields (×400). A specimen was regarded as positive when more than 10% of the neoplastic cells exhibited bcl-2 immunoreactivity. p53 immunoreactivity and PCNA expression were evaluated using the Quantitative ER/PR Analysis software in the CAS 200 image analyser (Cell Analysis Systems, Elmhurst, Illinois,

Figure 1  Immunostaining of bcl-2 in superficial, grade I TCC (A, ×200; B, ×400; methyl green counterstain). Note the predominant distribution of cells with bcl-2 immunoreactivity near the basement membrane. Bcl-2 immunoreactivity is not present at the superficial differentiating layer.
staining kit (Cell Analysis Systems). The slides were analysed using the Cell Measurement Program software in the CAS 200 Image Analysis System. Incomplete and blurred nuclei were not evaluated. At least 250 well preserved nuclei were evaluated in each section. The DNA content of the cell at G0/G1 phase was defined as 7.18 pg using a DNA calibration slide supplied by the CAS 200 Image Analysis System. The DNA content of each cell was calculated using the following formula: DNA content = 667/74 x optical density (OD)/peak OD/7.18, where the peak OD was obtained from the control slide in each case. The 2cDI was calculated as the sum of squares of the differences between the DNA values of single cells (ci) and the 2c value, divided by the numbers of measured cells (n) \[2cDI = \frac{\sum (ci - 2c)^2}{n}\].

The 5cER was defined as the percentage of cells with aneuploid nuclear DNA content over 5c that was not an exponent of 2. The areas analysed for nuclear DNA content were the same as those evaluated histologically.

**STATISTICAL ANALYSIS**

All numerical data were expressed as mean (SD). The relation between bcl-2 immunoreactivity and the clinicopathological findings was evaluated statistically using the \(\chi^2\) test. The relation between bcl-2 expression and p53 immunoreactivity, PCNA expression, 2cDI, 5cER, and the MNA was analysed using the unpaired Wilcoxon test. To evaluate prognostic relevance, survival was computed from the time of surgery until death or date of last follow up. The Kaplan–Meier method was used for univariate analysis and the log rank test was used to assess differences between groups. A p value below 0.05 was regarded as statistically significant.

**Results**

**BCL-2 IMMUNOSTAINING**

Bcl-2 immunostaining was typically cytoplasmic. In normal transitional epithelium and superficial TCC bcl-2 immunoreactivity was observed at the basal layer, and not at the leading edge of the tumour (figs 1A and 1B). In invasive TCC, however, bcl-2 positive cells were rarely observed (fig 2A), p53 positive cells were diffusely distributed (fig 2B), and were rarely seen at the leading edge.

**BCL-2 EXPRESSION IN RELATION TO CLINICOPATHOLOGICAL FINDINGS (TABLE 2)**

Bcl-2 was expressed in 19 (24.7%) of 77 TCCs and in 74 (96.1%) of 77 normal samples of transitional epithelium. In all cases, bcl-2 immunoreactivity was more intense in normal transitional epithelium than in TCC. Bcl-2 immunoreactivity was inversely correlated with histological grade (p<0.05) and was not correlated with gender distribution, the pT category, disease progression, and prognosis.
Table 2  Bel-2 immunoreactivity in relation to clinicopathological findings

<table>
<thead>
<tr>
<th>Clinicopathological findings</th>
<th>n</th>
<th>Bel-2 positivity (n (%))</th>
<th>p value (χ² test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histology</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>normal transitional epithelium</td>
<td>77</td>
<td>74 (96-1%)</td>
<td><strong>p&lt;0.001</strong></td>
</tr>
<tr>
<td>TCC</td>
<td>77</td>
<td>19 (24-7%)</td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>male</td>
<td>58</td>
<td>13 (25-9%)</td>
<td><strong>NS</strong></td>
</tr>
<tr>
<td>female</td>
<td>19</td>
<td>4 (21-1%)</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥53 years</td>
<td>49</td>
<td>13 (26-5%)</td>
<td><strong>NS</strong></td>
</tr>
<tr>
<td>&lt;63 years</td>
<td>28</td>
<td>6 (21-4%)</td>
<td></td>
</tr>
<tr>
<td>Histological grade</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>8</td>
<td>5 (62-5%)</td>
<td><strong>p&lt;0.05</strong></td>
</tr>
<tr>
<td>II</td>
<td>40</td>
<td>8 (20-0%)</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>29</td>
<td>6 (20-6%)</td>
<td></td>
</tr>
<tr>
<td>pT category</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pTa</td>
<td>13</td>
<td>5 (38-5%)</td>
<td></td>
</tr>
<tr>
<td>&gt;pT2</td>
<td>43</td>
<td>10 (23-2%)</td>
<td></td>
</tr>
<tr>
<td>Disease progression</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>no</td>
<td>43</td>
<td>14 (32-5%)</td>
<td><strong>NS</strong></td>
</tr>
<tr>
<td>yes</td>
<td>34</td>
<td>5 (14-7%)</td>
<td></td>
</tr>
<tr>
<td>Prognosis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>alive</td>
<td>45</td>
<td>14 (31-1%)</td>
<td><strong>NS</strong></td>
</tr>
<tr>
<td>dead</td>
<td>32</td>
<td>5 (15-6%)</td>
<td></td>
</tr>
</tbody>
</table>

n = Number of patients.

Bcl-2 IMMUNOREACTIVITY IN RELATION TO P53 IMMUNOREACTIVITY, PCNA EXPRESSION, 2cDI, 5cER, AND MNA (TABLE 3)

Bcl-2 immunoreactivity was inversely correlated with p53 immunoreactivity and was not correlated with PCNA expression. P53 immunostaining was observed in 39 (50-6%) of the 77 TCCs (>10% cells positive in each case). The 2cDI and 5cER were lower in bcl-2 positive compared with bcl-2 negative TCCs, but this difference did not reach statistical significance. The difference in the MNA between bcl-2 positive and negative TCCs also did not reach statistical significance.

BCL-2 IMMUNOREACTIVITY IN RELATION TO SURVIVAL (FIGS 3A AND 3B)

Using univariate analysis, there was no significant difference in survival rate between patients with bcl-2 positive and negative TCC. Patients with p53 positive TCC had a significantly worse clinical outcome compared with those with p53 negative TCC.

Discussion

Bcl-2 immunoexpression has been demonstrated in glandular epithelium of hormone and growth factor regulating tissues, such as prostate, breast and thyroid, as well as in epithelium containing longlived stem cells, such as the intestine. In the present study, in normal transitional epithelium and most superficial TCCs (pTa and grade I) bcl-2 immunoreactivity was observed at the basal layer and not in the upper differentiated layers of the epithelium. The characteristic distribution of bcl-2 immunoreactivity in transitional epithelium suggests that bcl-2 expression is present in undifferentiated cells undergoing terminal differentiation, which require protection from apoptosis. In most high grade, invasive TCCs bcl-2 immunoreactivity was not observed at the leading edge of the tumour and was inversely correlated with histological grade. These findings suggest that bcl-2 immunoreactivity is associated with a less aggressive TCC phenotype.

Bcl-2 immunoreactivity did not correlate significantly with cell proliferation (PCNA expression). But, bcl-2 may promote cell survival even when the cells are not proliferating and the part played by bcl-2 in the control of cell proliferation during disease progression, as has been found in some solid tumours, seems to be less important in TCC of the bladder.

Accumulation of p53 protein as a result of mutations in the p53 gene is associated with carcinogenesis and tumour progression, and potentially has an inhibitory effect on apoptosis. There is an inverse correlation between p53 and bcl-2 immunoreactivity in some solid tumours. In the present study p53 immunoreactivity was significantly associated with clinical outcome, whereas bcl-2 expression...
was not, suggesting that bcl-2 does not prolong cell survival in the transitional epithelium, with the associated risk of malignant transformation, and does not contribute to tumour progression in TCC of the bladder.

Cell proliferation is closely correlated with recurrence, invasion, metastasis, and prognosis in TCC of the bladder.25-27 Hemstreet et al.28 reported that the most important predictors of death and recurrence, respectively, in TCC of the bladder are stem line aneuploidy and the presence of cells with greater than 5c DNA. In the present study, we found no correlation between bcl-2 expression and indicators of genetic instability (5cER and 2cDI), suggesting that expression of bcl-2 has no effect on the behaviour of tumour cells in TCC of the bladder. Recently, Lipponen and Aaltomaa29 reported that apoptosis, evaluated by light microscopy and expressed as the number of apoptotic cells/mm² of neoplastic epithelium (apoptotic index), was independently related to recurrence-free survival in patients with superficial TCC of the bladder, but had no prognostic significance overall. Our results are very similar to those reported by Lipponen and Aaltomaa. We found no correlation between apoptosis, as evaluated by bcl-2 expression, and prognosis, and a strong correlation between bcl-2 expression and superficial TCC. In conclusion, bcl-2 expression in TCC of the bladder seems to be associated with a less aggressive phenotype and does not play an important role in tumour progression.

21 Bacus JW, Grade LJ. Optical microscope system for standardized cell measurements and analyses. Appl Optics 1987; 26:7280-93.
Immunohistochemical analysis of bcl-2 expression in transitional cell carcinoma of the bladder.

H Shiina, M Igawa, S Urakami, S Honda, H Shirakawa and T Ishibe

doi: 10.1136/jcp.49.5.395

Updated information and services can be found at:
http://jcp.bmj.com/content/49/5/395

*These include:*

**Email alerting service**
Receive free email alerts when new articles cite this article. Sign up in the box at the top right corner of the online article.

Notes

To request permissions go to:
http://group.bmj.com/group/rights-licensing/permissions

To order reprints go to:
http://journals.bmj.com/cgi/reprintform

To subscribe to BMJ go to:
http://group.bmj.com/subscribe/