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Immunohistochemical evaluation of p53, proliferating cell nuclear antigen (PCNA) and bcl-2 expression during bacillus calmette-guerin (BCG) intravesical instillation therapy for superficial bladder cancers

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Abstract Bacillus Calmette-Guerin (BCG) immunotherapy for superficial bladder cancer is now widespread, but non-effective cases are not uncommon and it has yet to be clarified why this is the case. In an attempt to cast light on this problem, we evaluated differences between effective and non-effective cases immunohistochemically using p53, proliferating cell nuclear antigen (PCNA), and bcl-2 antibodies. Between March 1988 and March 1996 a total of 79 superficial bladder cancer patients were treated with BCG intravesical instillation therapy after transurethral resection of bladder tumor (TUR-Bt). Of these, 19 demonstrated recurrence after the initial treatment. From the 60 remaining patients without recurrence, we randomly chose 19 additional cases and evaluated both series for p53, PCNA and bcl-2 immunohistochemical staining using formalin-fixed, paraffin-embedded tissues. For the recurrent cases, material taken prior and subsequent to BCG therapy was available for 17 of the 19 patients. Positive staining for p53 was noted for 42.1% (8/19) of both recurrent and non-recurrent cases, without any difference between the two. The rates for PCNA and bcl-2 were 52.6% (10/19) and 47.4% (9/19) in recurrent, and 36.8% (7/19) and 78.9% (15/19) in non-recurrent cases, respectively. Thus, there was a significant difference for lower incidences of bcl-2 in recurrent cases ($P = 0.044$). Values for p53 and bcl-2 were respectively 47.1% (8/17) and 41.2% (7/17) pre-treatment, and 52.9% (9/17) and 35.3% (6/17) post-treatment in the recurrence group. In contrast to the similarity in these results, PCNA positive cases were 52.9% (9/17) pre-treatment and 17.6% (3/17) post-treatment. These data suggest that there are differences between BCG-sensitive and BCG-resistant bladder cancers in terms of bcl-2 expression.

Key words BCG · Superficial bladder cancer · bcl-2 · Recurrence

Introduction

Bacillus Calmette-Guerin (BCG) immunotherapy for superficial bladder cancer is now widespread, but cases demonstrating recurrence are not uncommon and it has yet to be clarified why this is the case [1, 2, 9, 14, 16]. In an attempt to cast light on this problem, we evaluated differences between effective and non-effective cases immunohistochemically, concentrating on a number of parameters that have recently attracted a great deal of attention. The tumor suppressor, p53, was chosen since protein accumulation correlates with mutations as well as increase in urinary bladder tumor grade and stage [5], also being associated with proliferation [18] and disease progression [17, 21]. The nuclear factor known to inhibit apoptosis, bcl-2, was also selected for investigation since the apoptotic index appears to be positively related to mitotic activity in transitional cell carcinomas [10, 11]. Lastly, proliferating cell nuclear antigen (PCNA) expression, which has been found to be linked with invasion and recurrence of bladder tumors [15] was chosen as an indicator of the level of proliferation.

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Materials and methods

Between March 1988 and March 1996, 79 patients were treated at Nagoya City University Medical School with intravesical instillations of BCG (Tokyo 172 strain purchased from Nihon BCG Manufacturer, Tokyo) for superficial bladder tumors. The patients' ages ranged from 33 to 85 (average 65.5) years, and the male:female ratio was 69:10. All patients had a history of either multifocal or recurrent stage Ta to T1b papillary transitional cell carcinoma

(TCC) (Ta:T1a:T1b:Tx = 18:41:10:6) or carcinoma in situ (CIS, 4 patients) without any other concurrent malignancies or active tuberculosis infection. All the patients received BCG instillation treatments of 80 mg suspended in 40 ml of physiological saline, at 1-week intervals for the first six exposures and then at 1-month intervals for another six times as described previously [14]. BCG instillation treatments were started after transurethral resection of bladder tumor (TUR-Bt) exept four CIS cases. The time intervals between the tumor occurrence and BCG treatment were ranged from 2 to 4 weeks.

In all cases, specimens were obtained by cold cup biopsy before the treatment and fixed in 10% buffered formalin and embedded in paraffin, then sections were cut and stained with hematoxylin and eosin for light microscopic examination. Tumor grading and staging was performed using the "General Rules for Clinical and Pathological Studies on Bladder Cancer".

Of these 79 cases, 19 developed recurrence after the initial treatment. From the remaining 60 non-recurrent patients, we randomly chose 19 additional cases to constitute a non-recurrent group and evaluated both series for p53, PCNA and bcl-2 immunohistochemical staining using formalin-fixed, paraffin-embedded tissues. For the recurrent cases, materials taken prior and subsequent to BCG therapy were available for 17 of the 19 patients, allowing comparison of staining differences between the two groups.

Immunohistochemical methods

Immunohistochemical analyses were performed by a modified avidin-biotin-peroxidase technique [3]. Five-μm sections from paraffin-embedded blocks were deparaffinized using xylol and ethanol, and then treated as detailed below.

Analysis of p53 expression was conducted using mouse anti-p53 monoclonal antibody NCL-P53-D07 (Novocastra Laboratories, UK) which binds to both wild-type and mutant protein forms.

The slides were rinsed for 20 min with tap water, then treated with 0.05% protease for 10 min at room temperature, and washed with phosphate buffered saline (PBS) five times for 5 min each. The slides were covered with 0.1% H_2O_2 in PBS for 5 min at room temperature, washed five times for 5 min each with PBS and then, after immersing in 10% normal pig serum 20 min at room temperature to block nonspecific binding, were exposed overnight to mouse anti-p53 monoclonal antibody NCL-P53 D07 diluted 1:50 in PBS at 4°C, washed with PBS five times for 5 min each and incubated for 1 hour with horseradish peroxidase (HRP)-labeled goat anti-mouse immuno gamma globulin (IgG)γ + L IgGF(ab′)2 (TAGO Calif.) diluted 1:50 in PBS at room temperature. After further washing, binding sites were visualized by immersion in 3,3′-diaminobenzidine tetrahydrochloride/H_2O_2 solution for 5 min. The sections were then rinsed in tap water for 20 min, counterstained with hematoxylin, dehydrated, cleared and mounted.

For visualization of bcl-2 the same protocol was applied, with an additional autoclave step for 15 min at 121°C, immediately after deparaffinization, using the 124/M0887 monoclonal antibody diluted 1:40 (DAKO, Glostrup, Denmark). For PCNA immunohistochemistry, two 5-min microwave treatments were performed for initial antigen retrieval, and binding of the PC10/MO879 monoclonal antibody diluted 1:100 (DAKO) was demonstrated with routine methods.

Staining of sections was evaluated under a light microscope by one observer at objective magnifications of ×10, ×40 and ×100. The intensity of staining for all three parameters was graded subjectively into four categories as follows: 1, no positive staining; 2, slight or weak staining; 3, moderate staining; 4, heavy or intense staining. The extent of cellular involvement was similarly divided into four: 1, 0–24% of the cells; 2, 25%–49%; 3, 50%–74%; 4, 75%–100%. To obtain overall values the two figures were multiplied and a positive result arbitrarily defined as a product of 5 or more. Stromal reactions were ignored. To avoid differences in staining conditions, all the materials were stained simultaneously in one batch.

For statistical comparisons, incidence data were analyzed using NAP version 4.0 Mac 1994. $P < 0.05$ was considered statistically significant. The actual tests applied are given in Tables 1 and 2.

Results

The results for comparison of recurrent and non-recurrent groups are summarized in Table 1. There were no differences with regard to age, sex and number of previous recurrences, or in terms of grade or staging. Recurrence occurred between 1 and 41 months after BCG treatment.

Positive staining for p53 was noted for 42.1% (8/19) of both recurrent and non-recurrent cases, without any significant difference between the two. The rates for PCNA and bcl-2 were 52.6% (10/19) and 47.4% (9/19) in recurrent, and 36.8% (7/19) and 78.9% (15/19) in non-recurrent cases, respectively. Representative lesions, positive and negative for bcl-2 staining, are shown in Fig. 1. The incidence of recurrent cases positive for bcl-2 was lower, the difference being significant ($P = 0.044$). Data for the recurrent cases before and after BCG therapy are summarized in Table 2. Values for p53 and bcl-2 were respectively 47.1% (8/17) and 41.2% (7/17) pre-treatment, and 52.9% (9/17) and 35.3% (6/17) post-treatment, with no differences being apparent. In contrast, cases demonstrating positive PCNA scores decreased from 52.9% (9/17) pre-treatment to 17.6%

Table 1 Statistical comparisons of recurrent and non-recurrent groups for Bacillus Calmette-guerin (BCG) intravesical instillation therapy

	Recurrent group	Non-recurrent group	Significance (method)
Age	64.4 ± 12.0	65.2 ± 12.3	0.842 (t)
Sex (male vs female)	14 vs 5	15 vs 4	0.703 (χ^2)
No. of previous Recurrences	1.56 ± 2.28	1.22 ± 1.26	0.591 (t)
Grade	G1:G2:G3 = 5:13:1	G1:G2:G3 = 3:13:2	0.394 (U)
Staging	pTa:pT1a:pT1b =4:13:2	pTa:pT1a:pT1b = 5:8:5	0.734 (χ^2)
p53 positivity	8/19 (42.1%)	8/19 (42.1%)	1.000 (χ^2)
bcl-2 positivity	9/19 (47.4%)	15/19 (78.9%)	0.044 (χ^2)
PCNA positivity	10/19 (52.6%)	7/19 (36.8%)	0.511 (χ^2)

Positive results were defined as five or more points on multiplication of values for intensity of staining (1, no positive; 2, slight or weak; 3, moderate; 4, heavy or intense) and cellular involvement (1, 0–24% of the cells; 2, 25%–49%; 3, 50%–74%; 4, 75%–100%)
t, Student's t-test; χ^2, chi-square test; U, Mann-Whitney U-test; PCNA, Proliferating cell nuclear antigen

Table 2 Statistical comparisons of pre- and post-treatment groups for BCG intravesical instillation therapy

	Pre-treatment	Post-treatment	Significance (method)
Grade	G1:G2:G3 = 5:12:0	G1:G2:G3 = 6:11:0	0.734 (U)
Staging	pTa:pT1a:pT1b = 4:12:1	pTa:pT1a:pT1b:pT3a = 8:6:2:1	0.490 (χ^2)
p53 positive ratio	8/17 (47.1%)	9/17 (52.9%)	0.732 (χ^2)
bcl-2 positive ratio	7/17 (41.2%)	6/17 (35.3%)	0.724 (χ^2)
PCNA positive ratio	9/17 (52.9%)	3/17 (17.6%)	0.031 (χ^2)

Positive results were defined as five or more points on multiplication of values for intensity of staining (1, no positive; 2, slight or weak; 3, moderate; 4, heavy or intense) and cellular involvement (1, 0–24% of the cells; 2, 25%–49%; 3, 50%–74%; 4, 75%–100%)
χ^2, chi-square test; U, Mann-Whitney U-test

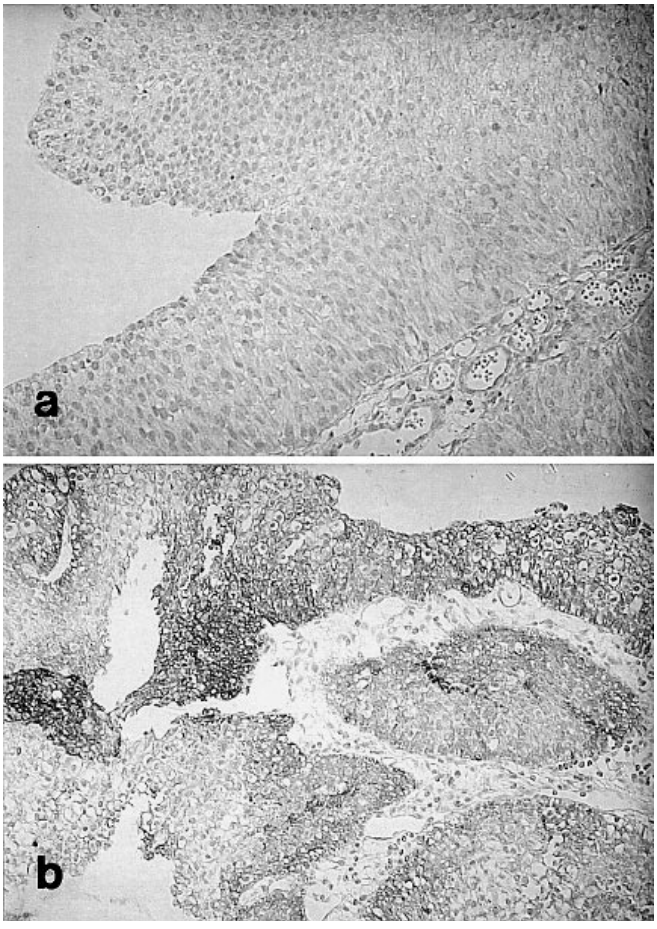

Fig. 1 Representative transitional cell carcinomas negative (**a**) and positive (**b**) for bcl-2. ABC method, ×150

(3/17) post-treatment ($P = 0.031$). No change in grading or staging was apparent.

Discussion

The present results indicate that whereas a lower level of bcl-2 expression is correlated with an increased risk of recurrence after BCG intravesical instillation therapy, p53 and PCNA are not informative parameters in this regard. Similarly the grade and stage of TCC does not appear to exert a significant influence on recurrence.

To our knowledge this is the first investigation of associations among p53, bcl-2 and PCNA in relation to BCG therapy. However, a large number of papers on their significance for urinary bladder tumor biology have recently appeared in the literature. In line with the present results, it has been found that p53 expression has no statistically significant link with recurrence after surgical resection [6, 20]. Furthermore, no relation to radiosensitivity of invasive bladder cancers was noted [13] and it has been concluded that utility of examining p53 expression for assessment of tumor biology is limited [19].

With regard to the present data for bcl-2 it is well recognized that this factor protects cells against apoptosis [7, 8]. Thus, its expression would be expected to promote growth of tumors. Therefore the present finding of a significant link between lowered bcl-2 expression and an increased risk of recurrence is perhaps surprising. While there was no evidence that the recurrent cases had less differentiated or more malignant lesions, the data suggest that a relative lack of bcl-2 might correlate with a higher resistance to BCG therapy. The recent paper by Masuda et al. [12] showing that a high apoptotic index is associated with a poor prognosis of TCC is naturally of interest in this context. While Glick et al. [6] found no link with prognosis, Daas et al. [4] also provided evidence that expression of bcl-2 protein is more common in low grade than in high grade TCC. This question clearly warrants further investigation, especially since so little is known about the role of bcl-2 and apoptosis in progression of bladder tumors.

While PCNA labeling was not significantly associated with the likelihood of recurrence in the present series of cases it did show a decrease after BCG treatment in those cases for which materials were taken both before and after therapy. What significance this finding may have, given the lack of any correlation with tumor grade or stage, remains unclear.

These data suggest that there are differences between BCG sensitive and resistant bladder cancers in terms of bcl-2 expression, so that this parameter may find application in prediction of individuals at particularly high risk of recurrence.

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