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RAPID COMMUNICATION

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Reciprocal expression of bcl-2 and p53 oncoproteins in urothelial dysplasia and carcinoma of the urinary bladder

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Abstract In order to investigate if and when the bcl-2 oncoprotein is activated in bladder tumorigenesis and its relationship with p53 overexpression and patient survival, we studied bcl-2 and p53 expression immunohistochemically in matched normal urothelium, dysplasia and cancer specimens selected by step-sectioning from 54 radically resected bladders for non-metastatic transitional cell carcinoma (TCC). In normal urothelium and mild dysplasia, bcl-2 was restricted to the basal cell compartment, while in moderate and severe dysplasia its expression was detectable also in the upper regions. Excess bcl-2 immunoreactivity was found in 27 (50%) of carcinomas, and a larger proportion of high-grade TCCs showed bcl-2 expression compared with that of low-grade TCCs ($P < 0.05$). Overexpression of p53 protein showed a increasing trend toward the progression of bladder tumorigenesis ($P < 0.01$) and a significant reciprocal correlation was found between bcl-2 and p53 expression in either various dysplasias ($P < 0.01$) or carcinoma ($P < 0.05$). With the evolution from mild dysplasia to carcinoma in individual cases, loss of bcl-2 expression was more frequently observed in superficial ($P < 0.02$) or low-grade carcinoma ($P < 0.05$) than in muscle-invasive or high-grade carcinoma. Furthermore, patients with negative immunostaining for both bcl-2 and p53 in cancer lesions had a significantly more favorable prognosis compared with those with positive immunostaining for the oncoproteins ($P < 0.05$), although bcl-2 by itself did not predict patient survival. We suggest that aberrant activated bcl-2, which is seen earlier than p53, appears to facilitate bladder tumorigenesis and to enhance tumor aggression in some extent.

Key words Bcl-2 · P53 · Immunohistochemistry · Urothelial dysplasia · Bladder cancer

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Introduction

Transitional cell carcinoma (TCC) of the urinary bladder is the twelfth most common cancer globally [8], and can be separated clinically into two separate entities, superficial and muscle-invasive TCC. At diagnosis, 80% of bladder cancers are superficial, i.e., they are limited to the urothelium (Ta) or infiltrate no deeper than the lamina propia (T1), which has a better prognosis [16]. The important features of superficial bladder cancer are its multifocal nature and high recurrence rate; also, many reports have indicated the presence of widespread dysplasia in normal-looking bladder mucosa in association with overt bladder cancer [6]. The concomitant dysplasia lesion has been considered to represent the "field change" of the urothelium. This hypothesis suggests that a carcinogenic insult initiates a first step in which the field of normal-looking mucosal cells has an increased potential to undergo a second step in which independent clones of cells result in the formation of multiple new tumors [12]; it, also provides an ideal model to study the proposed multiple-step nature of bladder tumorigenesis.

Recent studies have associated both the p53 and bcl-2 genes with the process of apoptosis. Although the exact mechanism for how these proteins interact in the pathway leading to cell death and tumorigenesis is not yet fully understood, it has been observed that bcl-2 can inhibit apoptosis triggered by wild-type p53 [18], and in contrast, the p53 gene product can also downregulate

bcl-2 gene expression [10]. An inverse relationship between p53 and bcl-2 has been reported in breast and colorectal cancers [4, 19], while this relationship is weaker in other carcinomas [15]. However, no study has addressed the relationship between bcl-2 and p53 expression in bladder tumorigenesis. Therefore, we analyzed bcl-2 expression in matched normal urothelium, dysplasia and cancer specimens selected by step-sectioning from 54 radically resected bladders for non-metastatic TCC, and determined the timing of bcl-2 activation and its relationship to p53 alteration and patient survival.

Material and methods

Sample selection

A total of 54 bladder TCC specimens with concomitant urothelial dysplasia lesions were selected by step-sectioning [3] from 121 resected bladders taken from patients with non-metastatic TCC by radical cystectomy at Fukui Medical University Hospital and Maitsuru Kyosai Hospital of Japan. The characteristics of the patients are summarized in Table 1. The diagnosis and grading of tumors were based on the "General Rules for Clinical and Pathological Studies on Bladder Cancer" (Japanese Urological and Pathological Association, 1993). The dysplasia lesions were classified into three grades: mild, moderate and severe, according to these criteria [10].

Immunohistochemistry

The following primary antibodies were used: murine IgG1 anti-bcl-2 monoclonal antibody (bcl-2/124, dilution 1:60, Dako, Glostrup, Denmark) and murine IgG2a anti-p53 monoclonal antibody (DO-1, dilution 1:60, Oncogene Science, Cambridge, Mass). The specificity and characterization of these two antibodies have been described previously [14, 17].

All immunohistochemical examinations were performed by a standard avidin-biotin-enhanced immunoperoxidase technique using a streptavidin biotin complex peroxidase kit (LSAB kit, Dako, Carpinteria, Calif.). Briefly, 4-μm-thick serial sections from archival formalin-fixed, paraffin-embedded tissue were placed on Silanized slides (Dako) and then air-dried overnight at 40 °C. After dewaxing in fresh xylene for 10 min, rehydrated through a graded alcohol series and transferred into PBS (pH 7.6), sections were then rinsed in methanol containing 0.3% hydrogen peroxide for 15 min to block endogenous peroxidase activity. In order to enhance antigen exposure, the slides were microwaved at low power (500 W, ER-245, Toshiba, Tokyo) for p53 or autoclaved at 121 °C for bcl-2 in citrate buffer (pH 6.0) for 20 min. After cooling, all samples were routinely blocked for 20 min in 2% milk solution followed by blocking reagent (Dako-LSAB kit) for 15 min before the addition of antibody. Sections were then incubated with antibodies (p53 or bcl-2) at 4 °C overnight followed by a second biotinylated antibody and the ABC (Dako-LSAB kit) for 45 and 30 min, respectively. Careful rinses were done with several changes of PBS between each step of the procedure in all immunostaining. The color was developed with diaminobenzidine (DAB, Sigma, St Louis, M.) as chromogen, the sections were then lightly counterstained with hematoxylin. Negative controls were carried out by replacing the primary antibody with PBS.

Double immunostaining for bcl-2 and p53 was accomplished by first labeling for bcl-2 as described above and next immunostaining for p53 by using the DAB-cobalt solution (0.02% DAB in PBS containing 0.01% $CoCl_2$ and 0.002% hydrogen peroxidase) as chromogen. After staining for bcl-2, slides were rinsed in a glycine buffer (pH 2.2) with three changes for 60 min at room temperature to remove the residual antigen–antibody complex of bcl-2 before being immunostained for p53.

Immunostaining analysis

Semi-quantitative evaluation was performed twice by one of the authors (BL) with a 2-week interval. The staining pattern of bcl-2 was classified as follow: −, negative; +, weak; ++, strong. Intensity of bcl-2 immunostaining of lymphocytes infiltrating the stroma of the bladder lesions was used as an internal positive control. Sections in which lymphocytes were bcl-2-negative were rejected and restained. Staining by p53 was assessed as the number of positively stained cell nuclei: < 10% positively stained nuclei were considered as negative (−), 11%–30% as weak (+); 31%–50% as strong (++) and > 50% as very strong (+++). Specimens from colorectal tumors, which were known to have p53 overexpression, served as positive controls. The semiquantitative evaluation was shown to be highly reproducible since no divergent diagnoses was made in the second assessment.

Statistical analysis

Comparison of bcl-2 and p53 immunostaining in different lesions was made by the chi-squared test. The association between either bcl-2 or p53 with various clinicopathological factors was analyzed using the Pearson's correlation coefficient. Kaplan-Meier survival curves were generated and compared by the logrank test. Multivariate analysis was conducted by the Cox model. Statistical significance was defined as $P < 0.05$.

Table 1 Clinicopathological data

Variable	
Age at surgery (years): mean (range)	69.7 (53–91)
Sex: male/female	43/11
Tumor stage	
Superficial (pTa-pT1)	27
Muscle-invasive (pT2-pT4)	27
Tumor grade	
Low (G1 and G2)	22
High (G3)	32
Infiltration	
Lymph vessel	31
Microvascular	16
Lymph node metastases	9
Follow-up term (months): median (range)	37 (3–106)

Results

Expression of bcl-2 oncoprotein

Normal urothelium

In histologically normal mucosa, weak bcl-2 immunostaining was observed in the basal cell compartment of the bladder urothelium in all tissue specimens but was absent in intermediate and superficial cell compartments (Fig. 1a). Very strong immunostaining of the cytoplasm and nuclear membrane of lymphocytes in the lamina propria was also observed in the normal area and in infiltrating lymphocytes within tumor stroma.

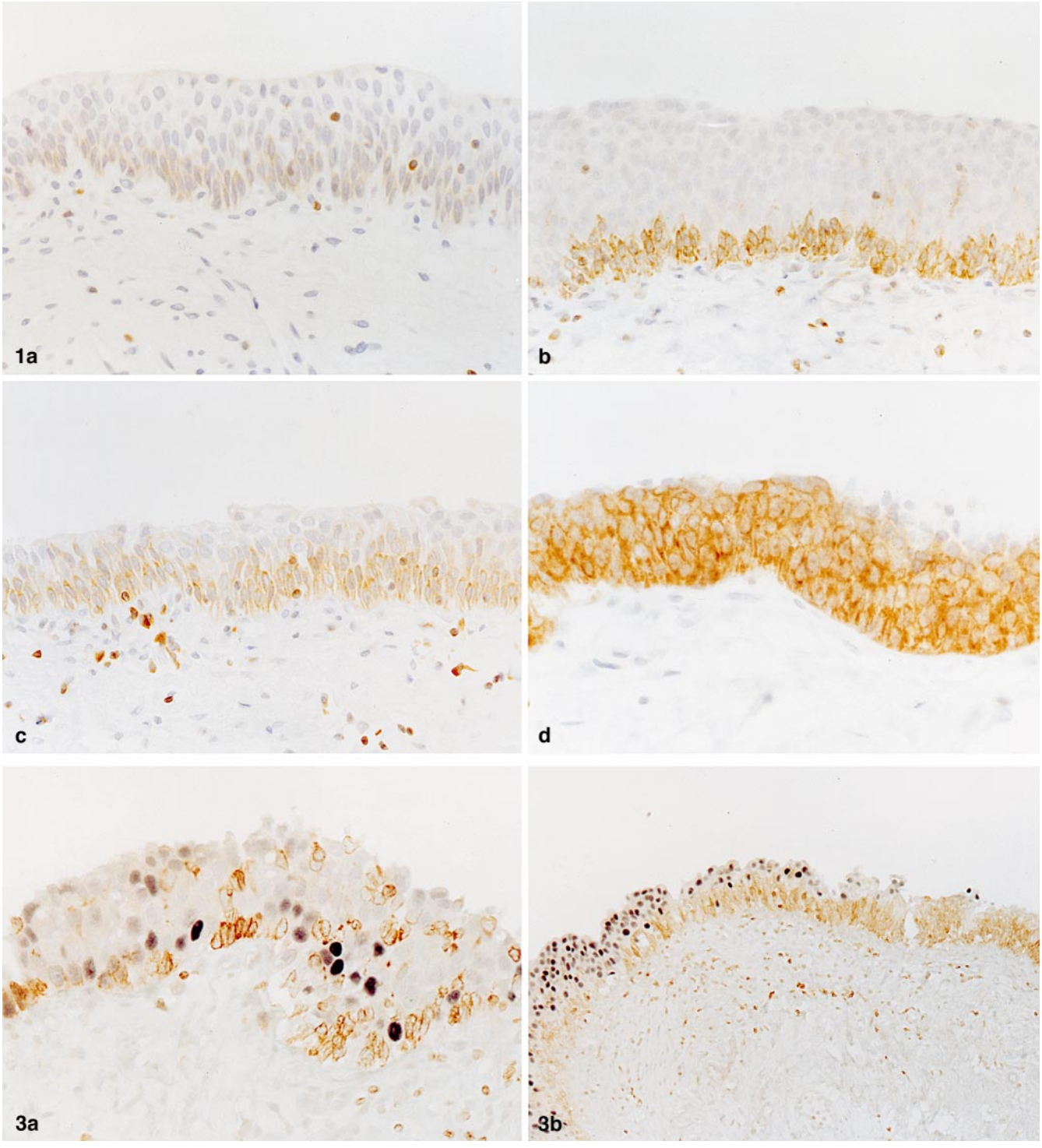

Fig. 1 Peroxidase immunostaining for bcl-2 expression in (**a**) normal urothelium, (**b**) mild dysplasia, (**c**) moderate dysplasia, (**d**) severe dysplasia. Original magnification ×100

Fig. 3a, b Double immunostaining for bcl-2 (brown) and p53 (darkblue) in specimens which contained immunoreactivity of bcl-2 and p53. (**a**) In TCC lesion, cancer cells were stained positively for bcl-2 but weakly or entirely negative for p53 and vice versa, ×150. (**b**) A clear change of bcl-2 decreasing and p53 occurring was observed in the evolution from severe dysplasia to carcinoma; ×75

Dysplasia

In 52 (96.3%) cases of mild dysplasia, bcl-2 immunoreactivity was found mainly in the basal cell compartments (Fig. 1b). Occasionally the suprabasal cells showed very weak immunostaining for bcl-2. In 45 (83.3%) cases of moderate dysplasia, bcl-2 expression was also found in the basal cell compartment, while in 19 of these 45 cases the intermediate cell compartment showed weak immu-

nostaining of bcl-2 in some cells (Fig. 1c). In 43 (79.6%) cases with severe dysplasia, bcl-2 expression was found in the basal cell compartment. Of these 43 cases, 28 showed bcl-2 expression in the intermediate cell compartment while bcl-2 was detected throughout the full thickness of the lesion in 4 cases (Fig. 1d). A significant raise of bcl-2 immunoreactivity in upper cell compartments was found with increasing severity of urothelial dysplasia ($P < 0.01$).

Carcinoma

A total of 27 (50%) TCCs had bcl-2 expression (Fig. 1d), which was not related to the histological stage of progression, lymphovascular infiltration or node invasion. However, a larger proportion (62.5%) of high-grade (G3) TCCs contained areas of bcl-2 immunostaining compared with 31.8% of low-grade (G1 and G2) TCCs ($P < 0.05$).

Expression of p53 oncoprotein

Normal urothelium

Normal mucosa was uniformly negative for p53 expression.

Dysplasia and carcinoma

Nuclear p53 positive immunostaining was restricted to dysplastic or cancer cells. There was a significant increase in either the average percentage of p53 positive cells or the number of p53 positive cases (as shown in Fig. 2) in the evolution from mild dysplasia to carcinoma ($P < 0.01$). A significant correlation was found between either p53 overexpression and histological stage ($r = 0.336$, $P < 0.05$) or cellular grade ($r = 0.408$, $P < 0.02$), but not with lymphovascular infiltration or node status.

Reciprocal expression of bcl-2 and p53 oncoproteins

There was a significant inverse correlation between bcl-2 and p53 immunostaining in various dysplasia lesions and TCCs, although an immunoreactivity for both bcl-2 and p53 was found in some cases, as summarized in Table 2. Of those specimens with both bcl-2 and p53 immunoreactivity, double immunostaining showed that the areas of immunoreactivity for these two proteins were topographically distinct in the majority of the specimens. Only three (5.6%) cases of severe dysplasia and two (4.2%) carcinomas expressed both bcl-2 and p53 in more than 50% of dysplastic or carcinoma cells. As shown in Fig. 3, although a few cancer cells stained positively for both bcl-2 and p53, the cells with most intense p53 immunoreactivity stained either weakly or negatively for bcl-2 and vice versa.

Changes of bcl-2 and p53 expression in the dysplasia–carcinoma sequence.

When analyzing the data by individual case, a higher proportion of loss of bcl-2 expression (from positive in dysplasia to negative in carcinoma) was found in superficial ($P < 0.02$) or lower-grade ($P < 0.05$) TCC cases than those in muscle-invasive or high-grade TCC cases, respectively. In contrast, the change in p53 overexpression from negative in dysplasia to positive in carcinoma showed an inverse relation with bcl-2 loss, and no loss of p53 expression, comparable to that of bcl-2, was found in the sequence (Table 3).

Survival analysis

Kaplan-Meier survival curves showed that the bcl-2 positive versus negative curve was not statistically different when tested by the logrank test. However, p53 overexpression was a highly significant predictor of poor prognosis ($P < 0.05$). As depicted in Fig. 4, when examining the combination of bcl-2 and p53 immunostaining status, patients harboring bcl-2(−)/p53(−) exhibited a significant longer survival than those with

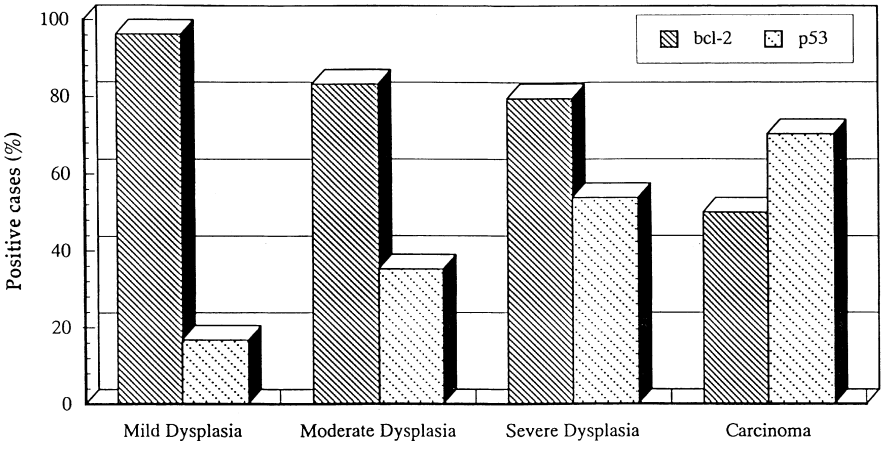

Fig. 2 Distribution of bcl-2 and p53 expression in dysplasia–carcinoma sequence. There was a significant difference in the alteration of either bcl-2 or p53 expression among the groups ($P < 0.01$)

Table 2 Combination of bcl-2 and p53 expression in dysplasia–carcinoma sequence

	n	bcl-2 (+)[a] p53 (+)[b]	bcl-2 (+) p53 (−)	bcl-2 (−) p53 (+)	bcl-2 (−) p53 (−)
Mild dysplasia	54	9 (16.7)	43 (79.6)*	0 (0)	2 (3.7)
Moderate dysplasia	54	14 (25.9)	31 (57.4)*	5 (9.3)	4 (7.4)
Severe dysplasia	54	19 (35.2)	23 (42.6)**	10 (18.5)	2 (3.7)
Carcinoma	54	19 (35.2)	8 (14.8)	19 (35.2)**	8 (14.8)

[a] bcl-2(+) included + and ++;
[b] p53(+) included +, ++ and +++
*$P < 0.01$; **$P < 0.05$

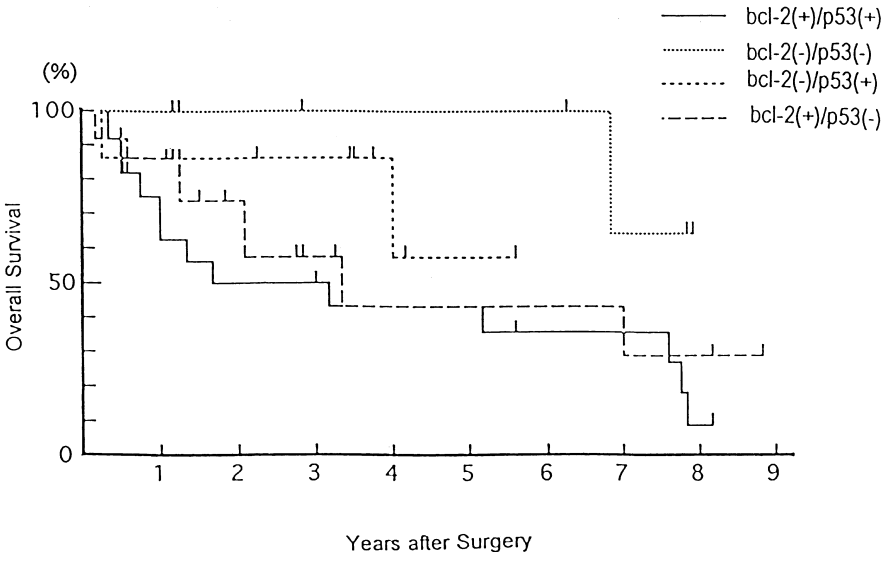

Fig. 4 Kaplan-Meier survival curves of patients categorized according to the combination of immunostaining for bcl-2 and p53 oncoproteins. There was a significant difference between bcl-2(−)/p53(−) and bcl-2(+)/p53(+) ($P < 0.05$, logrank test)

bcl-2(+)/p53(+) ($P < 0.05$), but no significant difference was found between other groups. In the multivariate analysis using the Cox model and including the parameters of age, tumor stage and grade, and bcl-2 and p53 expression, only tumor stage and grade as well as p53 expression proved to be independent prognostic factors ($P < 0.05$).

Discussion

Neoplastic transformation consisting of multistep accumulations of adverse genetic events occurs in a wide variety of human tumors over a large region of the genome. These genetic events include activation of dominant oncogenes by point mutations, gene amplification or rearrangement, and inactivation of tumor suppressor genes by point mutations or deletions. Over the past few years, bcl-2 has moved from being a molecule implicated in lymphoid translocation to being an important gene involved in key mechanisms in the pathogenesis of several types of neoplasm. In order to determine whether or not the bcl-2 oncoprotein is involved in bladder tumorigenesis, we analyzed bcl-2 expression in matched normal bladder urothelium, dysplasia and carcinoma. Our results indicated that bcl-2 oncoprotein was immunohistochemically detectable in the stem cell compartment of normal bladder epithelia, which reflects

Table 3 Alterations of bcl-2 and p53 expression in the evolution from dysplasia to carcinoma of the bladder

	n	bcl-2 loss[a] no. (%)	p53 occurrence[b] no. (%)
Tumor stage			
pTa-pT1	27	18 (66.7)	7 (25.9)
pT2-pT4	27	8 (29.6)	17 (62.9)
P value		<0.02	<0.02
Tumor grade			
Low (G1 + G2)	22	15 (68.2)	5 (22.7)
High (G3)	32	11 (34.4)	19 (59.4)
P value		<0.05	<0.02

[a] bcl-2 positive in dysplasia but negative in carcinoma
[b] p53 negative in dysplasia but positive in carcinoma

the progenitor cell role of basal cells requiring the protection of bcl-2 against apoptotic cell death to ensure survival of the entire epithelium, and its absence in the suprabasal layers of normal urothelium demonstrated that bcl-2 is not necessary during completion of the differentiation process. This was in broad agreement with recent studies of bcl-2 expression in normal urothelial, colorectal and cervical epithelia [2, 5, 7].

Although there were many reports concerning gene alterations and protein expression of bcl-2 in various cancers, little is known about bcl-2 expression in bladder cancer, particularly for the progression of bladder tumorigenesis. From our results in this study, with the

progression of urothelial dysplasia, a significant increase of bcl-2 expression in upper cell compartments was observed. An enhanced level of p53 overexpression was also found in the evolution from mild dysplasia to carcinoma. A similar staining pattern of bcl-2 protein has been reported in human cervical, colorectal and gastric dysplasias [5, 7, 9]. It might be hypothesized that bcl-2 expression restricted to the basal layer in mild dysplasia indicates that this proto-oncogene imparts only limited protection against cell death, and the same explanation might be proposed for the moderate dysplasia lesions, which show a non-significant increase of bcl-2 immunostaining in the upper layer of the lesions when compared with mild dysplasia. In severe dysplasia, the immunoreactivity of bcl-2 in the upper layer increased significantly and in some cases it extended into the full lesion. The more extensive expression of bcl-2 and p53 oncoproteins in the higher degree of dysplasia may indicate that an increased progressive capacity of the lesion is of fundamental importance in the development of a bladder cancer and that protection of urothelial cells against apoptosis may also play an important role in bladder tumorigenesis.

Furthermore, we observed a reciprocal correlation between bcl-2 and p53 immunostaining in various dysplasia and cancer lesions. During the progression from mild dysplasia to carcinoma, an overall positive rate of bcl-2 expression in this study began to decrease slightly in severe dysplasia while there was a significant increase of p53 overexpression in moderate dysplasia. Even in those cases which showed both bcl-2 and p53 immunoreactivity, double staining demonstrated that the areas of immunostaining for these two proteins were topographically distinct in the majority of specimens and only 5.6% of the severe dysplasias and 4.2% of carcinomas contained cells that identically expressed both proteins in more than 50% of dysplastic or malignant cells. A similarly inverse relationship between bcl-2 and p53 immunostaining has also been reported in human colorectal cancers [19]. These findings were in accordance with previous data that demonstrated some p53 mutants downregulate bcl-2 expression [4], and suggested either a potential downregulation of bcl-2 by abnormal p53 in the multistep process of TCC progression, or other factors accounted for the loss of bcl-2 expression. Moreover, it is possible that bcl-2 gene might contribute mainly to neoplastic transformation in the early stage of tumorigenesis by permitting dysplastic cells to persist until other synergistic oncogenes become activated, with the development of poor phenotypes because bcl-2 just promotes the short-term survival of hemopoietic cells after growth factor deprivation [13], and that high levels of bcl-2 protein might not be necessary to prevent apoptosis when tumors have acquired p53 mutations in major cancers.

The prognostic significance of bcl-2 expression in malignant tumors has attracted the attention of some authors, but it is controversial. Several recent studies suggested that bcl-2 expression is an independent prognostic factor associated with favorable clinical outcome in some human cancers including urinary tract cancers [1], but not in invasive bladder cancer [3]. In the present report, when analyzing the change of bcl-2 or p53 expression based on individual data of the cases, a higher proportion of loss of bcl-2 expression was found in superficial or low-grade cancers than in muscle-invasive or high-grade cancers during the evolution from dysplasia to carcinoma. The occurrence of p53 overexpression was more common in muscle-invasive or high-grade cancers than that in superficial or low-grade cancers. When looking at overall survival, Kaplan-Meier survival curves showed that the bcl-2 positive versus negative curve was not statistically different, and this was consistent with a recent study on invasive bladder cancer [3]. However, when the expression of bcl-2 and p53 proteins were taken together, patients who were negative for both bcl-2 and p53 expression showed a significantly longer survival than those with positive staining for the proteins. Thus, although bcl-2 by itself does not appear to have a major prognostic importance in bladder cancer, a persistent presence of excess bcl-2 protein might be associated with a poor prognosis to some extent. Aberrant expression of the bcl-2 gene and mutant p53 may enhance genetic instability individually by inhibiting apoptosis, thereby interfering with DNA impair processes and allowing further division of tumor cells containing genetic alterations necessary for tumor progression in more aggressive carcinoma.

Conclusion

We have showed that bcl-2 oncoprotein is expressed in a high proportion of urothelial dysplasia but is often lost in superficial or low-grade carcinoma; we have also demonstrated a reciprocal relationship between bcl-2 and p53 expression in dysplastic and cancer cells. Survival analyses indicate that the combined variable of bcl-2 and p53 is associated with prognosis, although bcl-2 alone does not relate to overall survival. Thus, our findings suggest that aberrant activated bcl-2, which might be earlier than p53, appears to facilitate an important role in bladder tumorigenesis and to enhance tumor aggression to some extent. Further studies investigating the expression of other bcl-2-related genes, the mechanisms regulating bcl-2 expression in bladder cancer and the functional interaction of bcl-2 with other oncoproteins involved in bladder tumorigenesis will be required before the role of bcl-2 in bladder cancer is fully understood.

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