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Correlations of Bcl-2 Expression with Clinicopathological Features in Breast Cancer

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To evaluate the prognostic significance of bcl-2, we investigated the correlation of bcl-2 expression with the established indicators of prognosis and tumor behavior in breast cancer. This study included a patient group of 91 histologically diagnosed female breast carcinomas. To determine the bcl-2 immunoreactivity, we used a monoclonal antibody directed against the bcl-2 protein by immunohistochemistry from paraffin-embedded tissue in a series of 91 women with breast cancer. Interpretable DNA histograms were obtained from 84 patients. The median age at diagnosis was 45.5 years and the median follow-up time was 30.5 months. Forty-eight (52.7%) cancers showed the bcl-2 immunoreactivity in the cytoplasm. The nonneoplastic portion of ductal epithelial cells and normal lymphocytes were usually stained with bcl-2 antibody. Estrogen receptors (ER)($p<0.001$) and progesterone receptors (PR)($p<0.001$) showed strong positive correlation with bcl-2 immunoreactivity. The histologic grade ($p<0.05$) and nuclear grade ($p<0.01$) also showed positive relationships with bcl-2 positivity but tumor size ($p>0.05$) and DNA ploidy ($p>0.05$) were not related with it. The bcl-2 positive patients showed longer survival ($p<0.05$) compared to bcl-2 negative tumors in univariate analysis (Kaplan-Meier life table analysis). Using multivariate analysis with Cox regression, bcl-2 ($p>0.05$), nuclear grade ($p>0.05$), ER status ($p>0.1$) and PR status($p>0.1$) were not reliable indicators for overall survival except histologic grade ($p<0.05$).

Our results suggest that bcl-2 expression may be related to hormonal regulation and tumor differentiation in breast carcinoma. Larger patient study groups with a longer follow-up period will be helpful to clarify the prognostic significance of bcl-2.

Key Words: Breast cancer, bcl-2 protooncogene

Current views of oncogenesis have focused mainly on abnormalities in cell proliferation, but the control of cell death has been recognized as one of the different pathways of carcinogenesis with the discovery of bcl-2 proto-oncogene which has functions in preventing apoptosis(programmed cell death) instead of promoting proliferation. In Korea, breast cancer is the third-leading female cancer and has been increasing steadily. The bcl-2 proto-oncogene was first identified because of its involvement in the t(14;18) chromosomal translocations found in many follicular B-cell lines and encodes 26-kD proteins (Yunis *et al.* 1982; Tsujimoto *et al.* 1984). The bcl-2 protein is located at the inner mitochondrial membrane, the nuclear envelope and the endoplasmic reticulum (Hockenbery *et al.* 1992), which grants a sur-

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vival advantage to breast cancer by inhibiting apoptosis(Korsmayer. 1992). It is well known that bcl-2 expression is found in tumors of hormonally responsive epithelium, such as prostate and breast(Colombel *et al.* 1993; Joensuu *et al.* 1994). We evaluated bcl-2 expression in breast cancer as a well known hormone responsive tumor by immunohistochemistry and correlation of bcl-2 expression with established prognostic factors.

MATERIALS AND METHODS

Patients characteristics

During the period from March, 1991 to February, 1996, 91 cases of female breast cancer were selected, based on the availability of paraffin-embedded tumor specimens at Yongdong Severance Hospital,Yonsei University. Seventy-seven out of 91 patients were infiltrating ductal carcinomas and 14 were ductal carcinoma in situ.

The median age at the time of diagnosis was 45.5 years and the median follow-up period was 30.5 months.

Histology

After fixation with 10% formalin, tumor tissues were embedded with paraffin, cut with 4 μm thickness and stained with hematoxylin-eosin. This slides were reviewed and evaluated for histologic classification by WHO classification and nuclear and histologic grading by the Bloom-Richardson numerical scoring system (Bloom and Richardson, 1957).

Immunohistochemistry of bcl-2

Paraffin sections were dewaxed in xylene and dehydrated through graded ethanol. For determination of bcl-2 expression, we observed cytoplasmic immunoreactivity with monoclonal mouse anti-human bcl-2 oncoprotein (clone 124, DAKO, Glostrup, Denmark). Bcl-2 positivity was scored by cytoplasmic staining as follows: grade 0- no staining, grade 1-slight staining in less than 10% of cells, grade 2-moderately strong staining in more than 10%, grade 3-strong staining present in almost all cells. Grade 0 and 1 were regarded as negative for bcl-2 and grade 2 and 3 were regarded as positive.

Steroid receptors

Estrogen receptor (ER) and progesterone receptor (PR) were determined using an enzymatic assay (Abbott Laboratories, Chicago, IL) and the amount of receptor protein per gram of tissue (fmol/mg). The positive cutoff value was greater than 20 fmol/mg.

Statistical analysis

Chi-square test was used to determine the pattern of association between bcl-2 and clinicopathological features. Survival and disease-free survival were determined by means of Kaplan-Meier method and examined by log rank test. For all statistical analysis, a P-value of less than 0.05 was considered statistically significant.

RESULTS

The pattern of immunohistochemical staining for bcl-2 is demonstrated in Figure 1 as cytoplasmic staining. Nonneoplastic breast tissues adjacent to the tumor and lymphocytes were usually positive for bcl-2. Of the 91 tumors, 48 (52.7%) were positive for bcl-2. The bcl-2 positivity in infiltrating ductal carcinoma was 49.4% (38/77) and in ductal carcinoma *in situ*, 71.4% (10/14) (Table 1). Estrogen and progesterone receptors showed strong positive correlation with bcl-2 immunoreactivity. Seventy-six percent of bcl-2 positive tumors were ER positive, whereas 24% of bcl-2 negative tumors were positive for ER ($p<0.001$). Progesterone receptor also showed a similar correlation with bcl-2 expression ($p<0.001$). The histologic and nuclear grade also showed a positive relationship with bcl-2 positivity ($p<0.05$, $p<0.01$), but tumor size, axillary node positivity and DNA ploidy were not related with it(Table 2). Bcl-2 positive patients showed longer survival ($p<0.05$) and disease-free survival($p>0.05$) compared to bcl-2 negative tumors in univariate analysis (Kaplan-Meier life table analysis)(Fig. 2, Fig. 3). Using multivariate analysis with Cox regression, bcl-2 ($p>0.05$), nuclear grade ($p>0.05$), ER status ($p>0.1$) and PR status ($p>0.1$) were not reliable indicators for overall survival except histologic grade ($p<0.05$). The patients with bcl-2 expression had favorable survival in univariate analysis but no significant survival benefit in multivariate analysis.

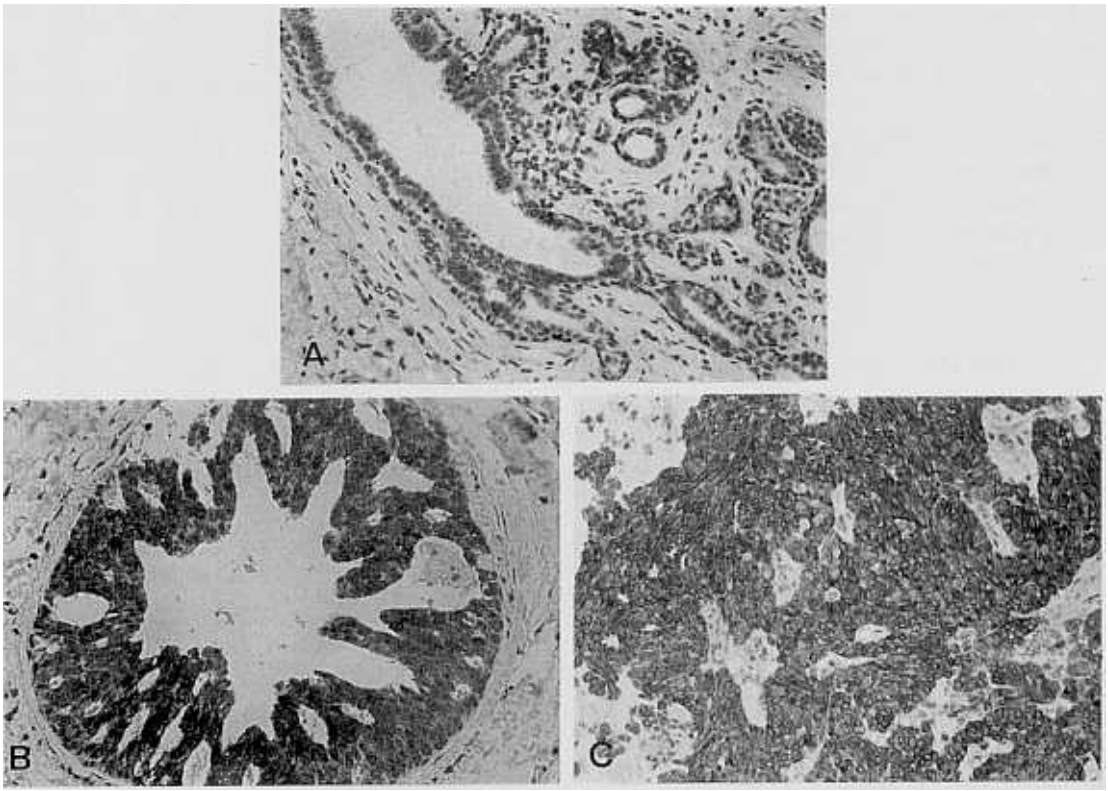

Fig. 1. Breast carcinoma cells with a positive cytoplasmic staining for bcl-2 :(A) Nonneoplastic breast tissues adjacent to the tumor, x200, (B) ductal carcinoma in situ, ×200 and (C) infiltrating ductal carcinoma, ×200.

Table 1. Bcl-2 expression in IDC and DCIS of the breast

bcl-2 expression		Number	%
IDC	positive	38	49.4
	negative	39	50.6
	total	77	100.0
DCIS	positive	10	71.4
	negative	4	28.6
	total	14	100.0

IDC : Infiltrating ductal carcinoma
DCIS : Ductal carcinoma *in situ*

DISCUSSION

Bcl-2 proto-oncogene was identified through investigations of a chromosomal translocation (14;18) frequently detected in follicular B-cell lymphoma (Yunis *et al.* 1982;Tsujimoto *et al.* 1984) and it encodes 26 kDa integral membrane oncoprotein which has been implicated in the control of cell death by blocking the process of programmed cell death (apoptosis), but does not effect the proliferation of cells (Hockenbery, 1992; Korsmayer, 1992). Recently, the regulation of cell death has emerged in the explanation of breast cancer cell growth along with the well-known investigations of breast cancer cell proliferation, and these appear to have correlations.

Immunohistochemical detections of bcl-2 expression were reported in many epithelial carcinoma such as nasopharynx cancer (80%)(Lu *et al.* 1993),breast cancer (75%)(Joensuu *et al.* 1994) and prostate cancer (76%) (Colombel *et al.* 1993). In this study, almost all normal breast tissues showed bcl-2 expression and breast cancer tissues showed bcl-2 positivity of 52.7%. Bcl-2 expression was more common in patients with ductal carcinoma *in situ* than those with infiltrating ductal carci-

Table 2. Correlation of bcl-2 protein expression with clinicopathological features in breast cancer

	bcl-2 negative N(%)	expression positive N(%)	p-value
Hormonal reactivity(n=87)			
ER positivity	10(24)	31(76)	<0.001
PR positivity	9(25)	27(75)	<0.001
Histologic grade(n=79)			0.03
Grade I	6(27)	16(73)	
Grade II	19(58)	14(42)	
Grade III	15(62)	9(38)	
Nuclear grade(n=79)			0.0027
Grade I	2(18)	9(82)	
Grade II	19(45)	23(55)	
Grade III	17(65)	9(35)	
Tumor size(n=87)			0.208
pT1	8(40)	12(60)	
pT2	22(45)	27(55)	
pT3	12(40)	18(60)	
Axillary node status(n=87)			0.241
positive	25(53)	22(47)	
negative	18(40)	26(60)	
DNA ploidy(n=84)			0.605
Diploidy	12(41)	17(59)	
Nondiploidy	26(47)	29(53)	

ER : Estrogen receptor
PR : Progesterone receptor
N : Number of patients

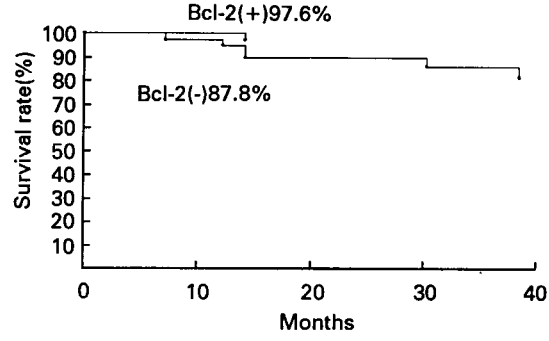

Fig. 2. Overall survival rate

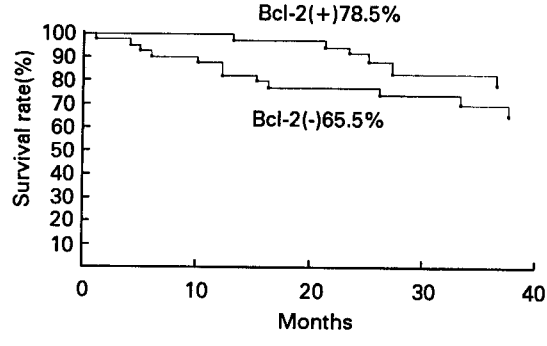

Fig. 3. Disease free survival rate

noma (71.4% vs 49.4%, p<0.05)(Table 1). These results suggest that the loss of bcl-2 activity may be related with tumor progression. Since infiltrating ductal carcinoma showed lower expressions of bcl-2 than ductal carcinoma *in situ* and much lower than normal breast tissue, the degree of bcl-2 expression and disease progression might have some correlation. In the analysis of the correlation between differentiation and bcl-2 expression, low histologic grade (HG) and nuclear grade (NG) (which indicate well-differentiated) showed high positivity of bcl-2 expression compared to high HG and NG. These results suggest that bcl-2 may suppress dedifferentiation in breast cancer and downregulate tumor progression and may be correlated with the assumption of the effect of bcl-2 on the growth, differentiation phenotype, and morphogenetic behavior of human mammary epithelial cells(Bhargava *et al.* 1994; Lu *et al.* 1995). It seems that the aggressive biologic behavior is related with the loss of bcl-2 expression, although there is no direct evidence of tumorigenic effect of bcl-2 in breast cancer.

Immunohistochemical studies on bcl-2 expression in human breast cancer have demonstrated a strong association with estrogen receptor status, illustrating the possibility that bcl-2 is an estrogen receptor-regulated gene (Bhargava *et al.* 1994; Leek *et al.* 1994). In this study, we also identified strong positive correlations between bcl-2 and estrogen receptors (p<0.05), as well as, progesterone receptors (p<0.05). The association between bcl-2 and estrogen receptor indicates a hormonal responsiveness and in a recent study, the up-regulation of bcl-2 gene expression is likely to be related to the estrogen dependent transcription pathways(Teixeira *et al.* 1995).

Regulation of apoptosis is a complex process and involves a number of genes including bcl-2, bcl-x, Bax and

related members (Boise *et al.* 1993). The correlation of bcl-2 and bcl-X_L has the effect on possible deregulation of apoptotic pathways and upregulation of antiapoptosis. Thus, it could play a role against endocrine therapy in c-erbB2 overexpression, ER positive cells associated with enhanced survival and breast cancer progression (Kumar *et al.* 1996). Generally, bcl-2 expression was absent in those tumors with expression of c-erbB2 and epidermal growth factor. Estrogen can promote resistance of estrogen receptor bearing human breast cancer cells to chemothereapeutic drugs through a mechanism that involves regulation of the bcl-2 proto-oncogene (Teixeira *et al.* 1995). Thus bcl-2 may affect the responsiveness to hormonal therapy and chemotherapy in breast cancer with the correlation of prognostic markers.

p53 has an inverse correlation with bcl-2 expression and mutant p53 may downregulate bcl-2 expression (Haldar *et al.* 1994; Joensuu *et al.* 1994). In the absence of lymph node metastasis, bcl-2 had a prognostic value on the relapse-free and overall survival with an inverse correlation between bcl-2 and p53. However, in the case of lymph node metastasis, p53 had a major prognostic role and weakened the prognostic effect of bcl-2 (Silvestrini *et al.* 1994; Silvestrini *et al.* 1996).

Recent studies have shown that the bcl-2 proto-oncogene suppresses programmed cell death (apoptosis) and that altered gene expression could enhance cell survival without affecting cell proliferation (Reed *et al.* 1987). We have observed longer survival in bcl-2 positive breast cancer than bcl-2 negative patients in univariate analysis but no survival benefit in multivariate analysis. And the loss of bcl-2 expression was somewhat linked to markers of poor prognosis such as positive Ki67, positive epidermal growth factor receptor (EGFR) and negative ER (Doglioni *et al.* 1994; Leek *et al.* 1994). Although the independent prognostic value of bcl-2 in breast cancer is still questionable, bcl-2 expression is associated with favorable clinicopathological features. And immunohistochemistry has limitations, such as the difference in positivity between studies due to intra- or inter-observer discrepancy and the selection of antibody. To clarify the prognostic significance of bcl-2 in breast cancer, the study of larger patients groups will be helpful.

In conclusion, bcl-2 expression seems to be more prevalent in well-differentiated tumors, suggesting that bcl-2 may suppress dedifferentiation of tumors and bcl-2 expression may be related to hormonal regulation in breast carcinoma. The prognostic significance of bcl-2 in breast cancer is uncertain but may be associated with a favorable prognosis and bcl-2 expression may be a marker of favorable response to endocrine therapy.

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