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ARTICLE

Down Regulation of Bcl2 Expression in Invasive Ductal Carcinomas Is Both Estrogen- and Progesterone-Receptor Dependent and Associated with Poor Prognostic Factors

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In normal breast, Bcl2 is expressed in the non-pregnant and non-involuting mammary epithelium. The exact mechanism and the effect of the down regulation of the Bcl2 expression on breast cancer cells are not clearly defined. We compared down regulation as well as the persistent expression of Bcl2 with ER, PR, p53, and c-erb-B2 overexpression and clinicopathologic variables, and tumor stage in 11 cases of ductal carcinomas in situ (DCIS) and 44 cases of invasive ductal carcinomas (IDC) of Korean women by immunohistochemical studies. Bcl2 down regulation was found in 39% of IDC and in 18% of DCIS cases. In IDC, while persistent Bcl2 expression was displayed in 95% and 78.9% of ER and PR immunoreactive ones and 71.9 % of c-erb-B2 immnonegative ones. Seventeen cases of Bcl2 down regulated IDC had a significant correlation with ER negativity (94.1%), PR negativity, (76.5%), and high nuclear (61.1% is grade III) and histological grade (76% is grade III). However, in DCIS, no significant correlation between the Bcl2 expression and various parameters were obtained, probably due to small sample size. In conclusion, the Bcl2 expression was both ER and PR dependent and down regulation of Bcl2 in IDC was significantly correlated with poor prognostic factors. (Pathology Oncology Research Vol 8, No 1, 26–30, 2002)

Keywords: Bcl2, estrogen receptor, invasive ductal carcinoma, ductal carcinoma in situ, progesterone receptor

Introduction

In breast cancer research, there have been many studies to determine prognostic markers. Recently, the regulators of the cell cycle and apoptosis have been the focus. Appropriate regulation of the apoptosis (or programmed cell death), is an important principle for the control of normal cell numbers and/or the removal of aged, aberrant or autoimmune cells during development, differentiation and maintenance of cells and organ systems.[1]

Antiapoptotic Bcl2 protein prolongs survival of the noncycling cells and inhibits cycling cells.[2] During the developmental period, Bcl2 is expressed in every tissue, however, in adults, it is expressed only in proliferating or reserve cells.[3] In normal adult state cells, Bcl2 makes heterodimer with a proapoptotic member of the Bcl2 family, i.e., Bax, but in proapoptotic conditions, intracytoplasmic Bax overexpression makes the equilibrium shift to the Bax homodimer; Bax forms a channel in the mitochomdrial membrane that allows the exit of cytochrome C, resulting in apoptosis. Whereas in apoptotic conditions, the equilibrium shift to the Bcl2 homodimer blocks the channel-forming activity of Bax. Proliferating cells overexpressing Bcl2 resist DNA damage-induced apoptosis, but undergo growth arrest in G0/G1 or in G2M, which promotes tumor cell survival and oncogenic process, but does not enhance cell proliferation.[4-6]

In the mammary gland, Bcl2 protein is expressed in the normal ductal epithelia of a nonpregnant and noninvoluting female.[7] Therefore, its expression in ductal carcinomas is a persistent expression or up-regulation and its absence indicates down regulation. In breast cancer cells, Doxorubicin-induced apoptosis occurred in Bcl2 negative cell lines, but not in Bcl2 positive ones.[6] The Bcl2 protein expressing cancers also show chemoresistance as well as extended sur-

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Table 1. **The summary of the clinical findings according to the Bcl2 expression**

	Bcl2	*No. of Cases (%)*	*Mean age (years)*	*Mean size of the tumor (cm)*	*Stage 0*	*I*	*IIA*	*IIB*	*IIIA*
DCIS									
(11 cases)	+	9 (81.8%)	41.4	2.91	9				
	–	2 (18.2%)	56	0.8	2				
IDC									
(44 cases)	+	27 (61.4%)	44.9	2.39		10	9	6	2
	–	17 (38.6%)	48.9	2.88		3	4	8	2

vival,[8,9] but they were associated with various favorable prognostic factors.[10-15] The Bcl2 expression was positively correlated with slow proliferation,[4,11,16] whereas it was negatively correlated with increasing histological grades and Ki67 labelling indices.[10,11,13,15] In more studies, Bcl2 expressing breast cancer cells showed increased doubling time, decreased S phase fraction, and increased G1/G0 fraction.[6]

According to Gasparini et al,'s study,[14] in contrast to in vitro data on drug resistance, Bcl2 expression was associated with better outcomes in patients treated with combined hormone and chemotherapy. They concluded that the expression of Bcl2 protein by the tumor cells might be a useful tool to distinguish patients for whom conventional forms of adjuvant therapy are beneficial from those with Bcl2 negative and ER-negative tumors for whom novel therapeutic strategies are needed.

In a total of 55 cases of breast carcinomas, including 11 cases of DCIS and 44 cases of IDC, we compared bcl2 down-regulation, as well as persistent expression, with ER, PR, p53, c-erb-B2 expression and four clinicopathologic variables of nuclear grade, histologic grade, lymph node metastasis and tumor stage.

Materials and Methods

A total of 55 cases of breast carcinoma specimens (11 cases of DCIS and 44 cases of IDC), obtained by modified radical mastectomy, were collected from the files of the Department of Pathology, Ilsan Paik Hospital, Inje University, College of Medicine from December 1999 to June 2001. All available clinicopathologic information was reviewed (*Table 1*) and all patients were Korean women.

Authors reviewed all available pathology slides of the 55 cases; World Health Organization (WHO) histologic classification of the breast tumors and three-tiered nuclear and histologic grading system were employed according to the Bloom and Richadson grading system. All representative cold 10% buffered formalin fixed and paraffin embedded specimens were cut to 2-3 μm thickness. Monoclonal antibodies for Bcl2 (Zymed, San Francisco, USA), ER (Immunotech, Marseille, France), PR (Immunotech, Marseille, France), p53 (Neomarkers, California, USA), and c-erb-B2 (Scytek, Utah, USA) were used (table 2). After deparaffinization and rehydration, the sections were subjected to a high temperature antigen unmasking process with a citrate buffer in an autoclave (121°C) for 20 min. After blocking in 2% skimmed milk, the sections were incubated with primary antibodies with ideal dilution for 60 minutes at room temperature. The sections were incubated with biotinylated secondary antibody, which was polyvalent and universal (prediluted, Immunotech, Marseille, France) and the expression was detected using the peroxidase labeled streptavidin biotin complex technique, according to the manufacturer's recommendations. Hematoxylin counter staining was performed. Appropriate positive controls were used (*Table 2*). For negative controls, primary antibodies were omitted. Cases were regarded positive when they showed either moderate or strong staining for these markers. Statistical analysis was made by chi-square test. We could not carried out correlation study with survival because of the short follow-up period.

Table 2. **Summary of primary antibodies used in this study**

Primary antibody	*Clonality*	*Dilution*	*Company*	*Control*
Bcl2	Monoclonal	1:50	Zymed (San Francisco, USA)	Lymph node
ER	Monoclonal	1:100	Immunotech (Marseille, France)	Breast cancer
PR	Monoclonal	1:50	Immunotech (Marseille, France)	Breast cancer
p53	Monoclonal	1:100	Neomarkers (California USA)	Breast cancer
C-erb-B2	Monoclonal	1:40	Scytek (Utah, USA)	Breast cancer
MIB-1 (Ki67)	Monoclonal	1:100	Immunotech (Marseille, France)	Lymph node

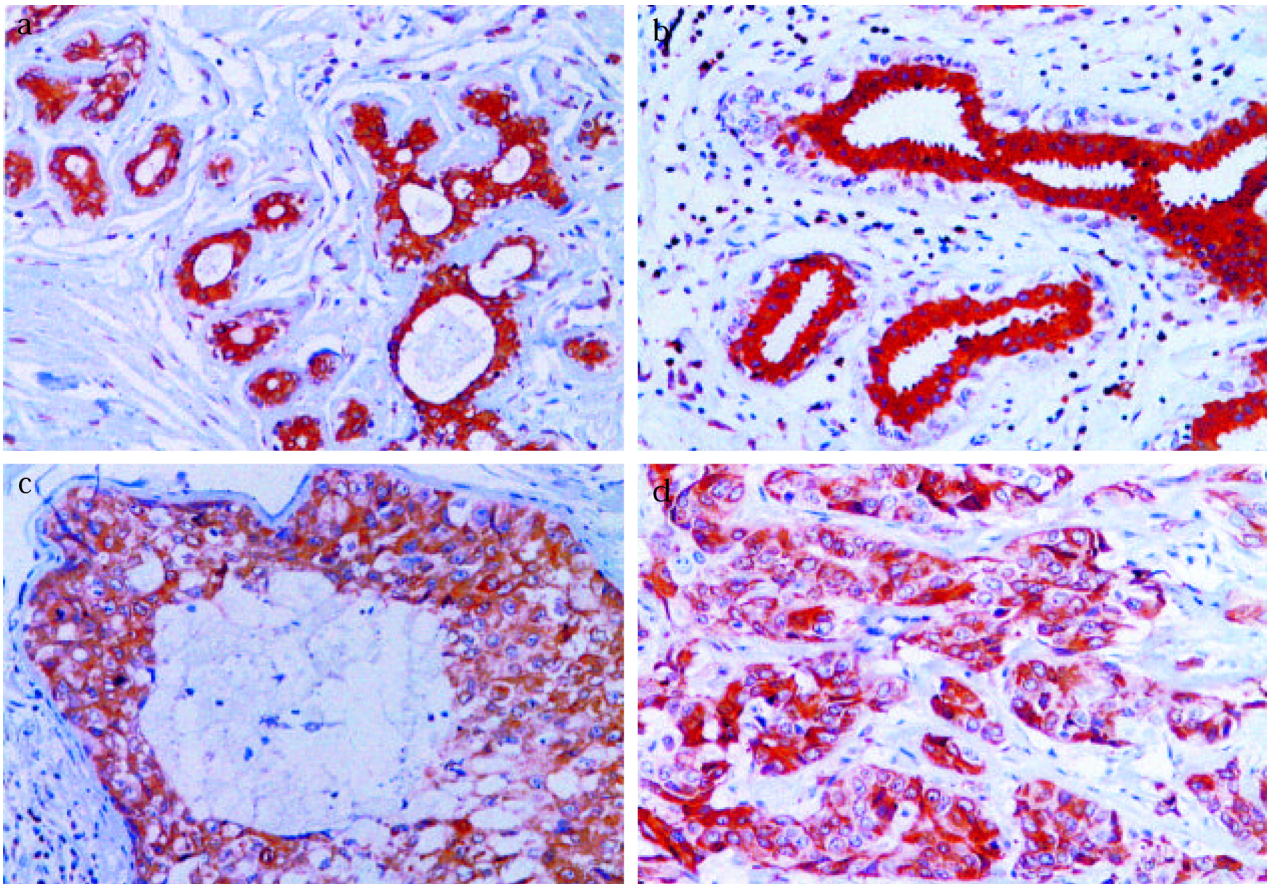

Figure 1. *Bcl2 immunostaining shows a robust expression in ductal epithelial and myoepithelial cells of normal breast (a), and epithelial cells of the fibroadenoma (b), DCIS (c) and invasive ductal carcinoma (d). Infiltrating lymphocytes are also immunoreactive for Bcl2 monoclonal antibody. In fibroadenoma, myoepithelial cells show weak or negative expression of Bcl2 (B).*

Results

In our study, the pattern of Bcl2 immunostaining was cytoplasmic and it was compatible with its localization in the outer membrane of the mitochondria.[5] All normal ductal epithelia and myoepithelia adjacent to the tumor showed robust Bcl2 expression (*Figure 1*). Bcl2 persistent expression was found in 9 (81.8%), and 27 (61.4%) cases of DCIS and IDC, respectively (*Figure 1*). ER immunoreactivity was seen in 6 (54.5%) and 20 (45.5%) cases of DCIS and IDC. PR immunoreactivity was seen in 6 (55%) and 19 (43%) cases of DCIS and IDC. P53 immunopositivity was noted in 3 (27.3%) and 14 (31.8%) cases of DCIS and IDC. C-erb-B2 expression was observed in 7 (63.6%) and 12 (27.3%) cases of DCIS and IDC. The results are summarized in *Table 3*.

In IDC, Bcl2 persistent expression was displayed in 19 (95%) out of 20 ER immunoreactive cases, 15 (78.9%) out of 19 PR immunoreactive IDC cases and 23 (71.8%) out of 32 c-erb-B2 immnonegative cases. Bcl2 persistent expression was found in 100% (2/2 case), 75.0% (18/24 cases) and 38.9% (7/18 cases) of nuclear grade I, II and III IDC, respectively. Therefore, Bcl2 persistently expressing IDC were associated with predominant expression of ER and PR and c-erb-B2 immunonegativity ($p<0.05$). They showed negative correlation with higher nuclear grade ($p<0.05$). However, they were not correlated with p53 expression, histological grade, or stage status ($p>0.05$). Additionally, they were not correlated with patient age and primary tumor size.

In contrast, Bcl2 down regulated IDC had an inverse correlation with ER (i.e., 94% is ER negative), PR (76.5% is PR negative), high nuclear (i.e., 61% is grade III), and histological grade (i.e., 76% is grade III) ($P<0.05$). More lymph node metastases (Bcl2 (-) cases: 65%, Bcl2 (+) cases: 44%) were found, but it was not statistically significant. Therefore, Bcl2 down regulating IDC were strongly correlated with poor prognostic factors. However, they were not correlated with p53, and c-erb-B2 expression or stage status ($p>0.05$). In DCIS, we could not find any significant correlation between Bcl2 persistence or down regulation with other parameters, which was probably due to the small sample size.

Table 3. **The immunoreactivity of the Bcl2, ER, PR, p53 and c-erb-B2 in 44 invasive ductal carcinomas**

	ER		*PR*		*p53*		*c-erb-B2*		
Bcl2	+ (%)	– (%)	+ (%)	– (%)	+ (%)	– (%)	+ (%)	– (%)	Total (%)
+	19	8	15	12	7	20	4	23	27 (61)
–	1	16	4	13	7	10	8	9	17 (39)
Total	20 (45)	24 (55)	19 (43)	25 (57)	14 (32)	30 (68)	12 (27)	32 (73)	44 (100)
p value*	p<0.01		p=0.049		p=0.329		p=0.024		

*; t test between Bcl2 positivity with other parameters including ER, PR, p53, and c-erb B2.

Discussion

Antiapoptososis is, also, a normal physiological process like apoptosis. The antiapoptotic protein, Bcl2, is normally expressed in some adult progenitor cells, such as blood cells and crypt epithelia of the intestine.[17] The breast is one of the few organs that complete its development after birth through the two physiological states, puberty and pregnancy. In breast tissue, Bcl2 is known to be expressed in the normal mammary epithelial cells of the non-pregnant female and during early pregnancy but undetectable in the lactating or involuting mammary gland.[7] A balance between cell proliferation and apoptosis controls the development and function of a normal breast.

In our study, Bcl2 was expressed in 100% of normal ductal epithelial and myoepithelial cells adjacent to the tumor and of the control cases. These results showed that the antiapoptotic function of Bcl2 is required in adult mammary glands, which always express estrogen receptor. However, prolonged survival without excessive proliferation is needed for homeostasis of the non-pregnant, non-lactating or non-involuting breasts. If, in addition to decreased cell death, excess cell division occurs, hyperplasia will be induced, and it may be a precancerous lesion of malignant transformation.[18] Bcl2 protein is known to promote cell survival, but does not proliferate the cells, and these two processes are subject to distinct genetic control.[1] Therefore, if breast cancer cells express Bcl2 protein, it may be persistent expression, and if not, it may be down regulation.

There have been studies about antiapoptotic Bcl2 and proapoptotic Bax expression in breast carcinomas.[6, 10-15] however, until now, we did not know the exact mechanism of the Bcl2 family in breast carcinonogenesis. In breast carcinomas, Bcl2 persistent expression was found in about 61-70% of invasive ductal carcinomas, 66% of micropapillary carcinomas[19] and 2.9% of apocrine carcinomas.[20] (*Table 4*). The expression of Bcl2 protein has been shown to suppress apoptosis in response to a number of stimuli, including anticancer drugs.[13] Therefore, Bcl2 may mediate chemoresistance in some patients and Bcl2 could be a target for the development of new anticancer therapy.[9] Inhibition of Bcl2 expression by antisense oligonucleotide (12-30 nucleotide)[9] or dominant negative inhibitor Bcl-xs has already been shown to promote apoptosis and to sen-

Table 4. **Percentage of expression in breast carcinomas**

Authors	*Materials (Number and type of breast cancer)*	*% of Bcl2 positivity*
Present data, 2002	44 invasive ductal carcinoma	61.4% (27/44 cases)
Leal et al., 2001[20]	35 apocrine carcinoma (22 pure & 13 with invasive carcinoma)	2.9% (1/35 cases)
Luna-More et al., 2000[19]	50 micropapillary carcinoma	66.0% (33/50 cases)
Mauri et al., 1999[12]	232 infiltrating ductal carcinomas 19 infiltrating iobular carcinomas 6 medullary carcinomas 9 infiltrating tubular carcinomas 5 mucinous carcinomas 6 oribriform infiltrating carcinomas	74.1% (140/189* cases)
Gasparini et al., 1995[1-4]	149 infiltrating ductal carcinomas 23 infiltrating lobular carcinomas 8 other types of carcinomas	65.0% (117/180 cases)

*among materials, the exact types of breast carcinomas, which was done Bcl2 immunohistochemistry, was not mentioned on original papers

sitize cells to chemotherapy-induced apoptosis.[21] Despite the antiapoptotic and chemoresistant effects favoring tumor survival, Bcl2 prolongs cell cycle and decreases tumor cell proliferation, and these functions may account for the association of Bcl2 persistent expression with favorable breast cancer outcomes.[11,14]

In our studies, persistent expression of Bcl2 was found in 61.4% of IDC. Like the results of the previous studies, Bcl2 expression was significantly associated with the favorable prognostic indicators, such as ER and PR expression, c-erb-B2 immunonegativity, and a low nuclear grade by the t-test ($p<0.05$). However, it did not show any correlation with p53 expression pattern, histological grade and tumor stage in IDC.

Conversely, Bcl2 down regulated IDC were strongly correlated with ER negativity (94.1%), PR negativity (76.5%), and higher nuclear (61.1%) and histologic grade (76%) ($p<0.05$). However, Bcl2 down regulation was not associated with p53 and c-erb-B2 expression.

In DCIS, Bcl2 persistent expression was higher (82%) than that (61.4%) of IDC, but we could not find any significant difference from other parameters, probably due to a small sample size.

Of the utmost importance is the association between Bcl2 expression and ER and PR status. Leung and Wang[22] found a paradoxical effect of 17-beta-estradiol on two anti-apoptotic proteins Bcl2 and Bcl-x. 17-beta-estradiol resulted in up-regulation of Bcl2 mRNA and protein, but down-regulated Bcl-X mRNA and protein. In addition, Tamoxifen, an anti-estrogen, blocked the down-regulation of Bcl-X with 17-beta-estradiol, demonstrating that this effect is estrogen dependent. These findings suggest that Bcl2 family proteins may be regulated through unique pathways and these pathways may be modulated by estradiol. Hence, the expression of the ER by the tumor cells controls the Bcl2 expression or down regulation.

In our study, Bcl2 expression in IDC was both ER and PR dependent. Paradoxically, the absence of the antiapoptotic oncogene product, Bcl2 was correlated with unfavorable prognostic indicators. For more clarification of the prognostic roles of Bcl2 persistence or down regulation, further correlation studies with survival are required.

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