Egyptian Journal of Surgery Vol 25, No 4, Oct., 2006

ORIGINAL ARTICLE

PROGNOSTIC SIGNIFICANCE OF BCL-2, BAX AND P53 EXPRESSION IN BREAST CANCER

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Aim: *of this study was to investigate the significance of apoptosis-related genes bcl-2 and Bax in breast carcinoma cases treated with radical surgery plus radiotherapy and adjuvant chemotherapy for at least 1 year and their relation with expression of p53.*
Methods: *After surgical resection immunohistochemistry was performed to determine Bcl-2, Bax, p53 and estrogen receptor (ER) expression in paraffin-embedded tissues of 50 invasive breast cancers.And overall survival was assessed.*
Results: *Bcl-2, Bax, P53 and ER immunostaining displayed a positive relation with increasing histologic grade (P=0.000) and negative relation with the staging .Bcl2 displayed a negative relation with p53 (P=0.035) and a positive relation with Bax and ER (P= 0.003 and P= 0.011 respectively). Expression of Bcl2 was associated significant improvement in overall survival (p=o.o1).*
Conclusion: *regulation of apoptosis is important in invasive ductal carcinoma. These results indicate that bcl-2 expression is significantly associated with hormonal receptor status so it is good prognostic marker, and that p53 is a significant prognostic marker. No significant relation between Bax and overall survival in relation to the stage, p53 or ER*

Keywords: *Carcinoma, Apoptosis, Prognosis.*

INTRODUCTION

Dysregulation of normal programmed cell death mechanisms plays an important role in the pathogenesis and progression of breast cancer, as well as in responses of tumors to therapeutic intervention. Overexpression of anti-apoptotic members of the Bcl-2 family such as Bcl-2 and Bcl-X(L) has been implicated in cancer chemoresistance, whereas high levels of pro-apoptotic proteins such as Bax promote apoptosis and sensitize tumor cells to various anticancer therapies. Though the mechanisms by which Bcl-2 family proteins regulate apoptosis are diverse, ultimately they govern decision steps that determine whether certain caspase family cell death proteases remain quiescent or become active. To date, approximately 17 cellular homologs of Bcl-2 and at least 15 caspases have been identified in mammals. Other types of proteins may also modulate apoptotic responses through effects on apoptosis-regulatory proteins, such as BAG-1-a heat shock protein 70 kDa (Hsp70/Hsc70)-binding protein that can modulate stress responses and alter the functions of a variety of proteins involved in cell death and division.(1)

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.(2) The expression of Bcl-2 protein has also been reported in breast cancer(3-5) without relation to tumor type. This protein is capable of preventing apoptosis and promoting tumor development. Leek et al(5) found a direct relation between Bcl-2 expression and the presence of estrogen receptors in breast carcinoma. These authors also noticed no relation between Bcl-2 protein expression and nodal status, tumor size, or differentiation. Others have reported strong Bcl-2 expression in small, estrogen-receptor-positive, slowly proliferating, and p53-negative tumors.(7) Interestingly, it has been demonstrated that in

human breast carcinoma cell lines, a mutated and/or wild-type p53 downregulates Bcl-2 expression.[8]

Bax is normally expressed in several epithelia including breast, small intestine, colon, prostate, respiratory tract and skin. Reduced expression in breast cancer is associated with poor response to chemotherapy and shorter survival[9]

Estrogen, by interacting with its receptor (ER), plays a central role in regulating the proliferation and differentiation of normal breast epithelium. During the last 10 years, many groups have used immunohistochemistry (IHC) to measure ER in breast cancers. These studies showed a 20-30% average reduction in recurrence/mortality in ER-positive patients receiving adjuvant endocrine therapy and an approximately 60% overall clinical response rate in patients with ER-positive advanced breast cancer treated with endocrine therapy. The primary reason for measuring ER in breast cancer today is this ability to predict response to endocrine therapy.[10,11] The aim: of this study was to investigate the significance of apoptosis-related genes bcl-2 and Bax in breast carcinoma cases treated with radical surgery plus radiotherapy and adjuvant chemotherapy for at least 1 year and their relation with expression of p53.

PATIENTS AND METHODS

This study included 50 female patients presenting with breast lump to general surgery department Mansura University over aperiod of 80months, between 1999 and 2005. All patients subjected to history taking, clinical examination, breast mammogram, ultrasound, and trucut needle biopsy. Modified radical mastectomy was done for all patients. Specimens were sent to histopathology department for assessment of stage, grade,and apoptotic figures as follow.

Immunohistochemistry: Three um thick, formalin fixed, paraffin embedded sections were immunostained. First, hematoxylin and eosin-stained histologic sections were performed to confirm the diagnosis. Then additional 3-5 micron thick sections were cut from the paraffin blocks for immunohistochimstery. Tissue sections were deparaffinized in fresh xylene and rehydrated to water. A biotin-streptavidin method was used as previously described[12,13] using Histostain-SP bulk for broad spectrum kit Zymed laboratories INC. Endogenous peroxidase activity was blocked using 3% hydrogen peroxide in methanol for 10 minutes. Blocking solution (10% non-immune goat serum) was applied for 5 minutes. Excess reagents were tapped off. Antigen retrieval was done, then the slides were cooled at room temperature (20 minutes). Followed by washing in distilled water and rinsed in phosphate-buffered saline (PBS). The primary antibody was done using primary antibodies to Bax (DAKO, Rabbit polyclonal, code NO A 3533) , Bcl2 (DAKO, Mouse monoclonal, code NO N1587), ER (Zymed, Mouse monoclonal, code NO 90245845), and P53 (DAKO, Mouse monoclonal, code NO N1581).

Staining characteristics & assessment of staining: ER & P53 staining was nuclear and were considered +ve when more than 10% of tumor cells were labeled. Bax and Bcl2 Staining was cytoplasmic and were considered +ve when more than 20% of tumor cells in bcl2 and 30% in bax were positive. For Bax & Bcl2, staining lymphocytes served as internal +ve control. Tonsil was also used as positive control for Bcl2 and known positive cases of breast and prostate cancer were used as +ve controls for ER and P53 respectively.

Labelling of these markers was assessed as negative (-) weak (+), moderate (++) & strong (+++). In most cases, the staining was homogeneous but in some tumors a more heterogeneous pattern was observed.[14]

Statistical analysis: Data were analyzed using SPSS (Statistical Package for Social Science) Version 10. Fisher's exact test and chi-square test were used for comparison between categorical variables. Kaplan-Meier survival analysis was to estimate overall survival and log-rank test was used for comparison of survival between groups. P value less than 0.05 was considered to be statistically significant.

RESULTS

Mean patient age was 58 years (range 29 to 79 years) with a median follow up of 80 months from time of diagnosis. The size of their tumors ranged from 0.6 to 5.0 cm (mean= 1.9; median= 1.7). The histopathology was infiltrating ductal carcinoma (Fig. 1). Using Elston and Ellis grading system and American Joint Committee on Cancer for staging, invasive ductal carcinomas were divided as grade (1) well differentiated (n=6), grade (2) moderately differentiated (n=39) and grade (3) poorly differentiated (n=5). Also staging were done as stage I (n =6), stage II (n=35), stage III (n =5) & stage IV (n=4).

In this study, the predominant intracellular distribution of Bcl2 was in the cytoplasm and or cell membranes (Figs. 2,3), but Bax was cytoplasmic (Fig. 4). P53 and ER were expressesd as nuclear staining (Figs. 5,6 respectively).

At follow-up, only 3 patients died of disease 36 months after surgery. Of the others, 16 were alive with disease (mean survival=43 months; median=46 months), and 31 were alive with no evidence of disease (mean survival=38 months; median=82 months).

Bcl2 expression displayed a significant relation with increasing histological grades Table 1. (P=0.000) and negative relation to stages of IDC, as in Table 2. (P=0.061) (chi-square test) It associated positively with ER (P=0.011)

and Bax (P=0.003) but negatively with P53 (P =0.035) Table 3. (Fisher's exact test).

Bax expression displayed a significant relation with increasing histological grades Table 1. (P=0.000) and negative relation to stages of IDC, as in Table 2. (P=0.175). It did not associate to p53 (P=0.163), but positive relation was seen with Bcl2 (P = 0.003) and ER (0.01) Table 4. (Fisher's exact test).

P53 expression displayed a significant relation with increasing histological grades Table 1. (P=0.000) and negative relation to stages of IDC, as in Table 2. (P=0.146).

ER expression displayed a significant relation with increasing histological grades Table 1. (P=0.000) and negative relation to stages of IDC, as in Table 2. (P=0.062).

Table5. displayed significant improvement in overall survival for patients with grade I and 1I IDC in relation to patients with grade III (P=0.001) Also significant improvement in overall survival for patients with positive bcl2 over the negative patients (p=0.01). (Kaplan-Meier survival analysis)

Table 1. cl2, Bax, P53 and ER expression in relation to grading of the tumor.

		Grade	I	Grade	II	Grade	III	Total		Chi-square test	
		No	%	No	%	No	%	No	%	value	P-value
Bcl2	**Negative**	0	0	3	6	10	20	13	26	54.9	0.000
	Mild	1	2	15	30	2	4	18	36		
	Moderate	2	4	8	16	0	0	10	20		
	Strong	8	16	1	2	0	0	9	18		
Bax	**Negative**	1	2	2	4	8	16	11	22	28.4	0.000
	Mild	1	2	10	20	2	4	13	26		
	Moderate	5	10	14	28	1	2	20	40		
	Strong	4	8	1	2	1	2	6	12		
P53	**Negative**	11	22	9	18	0	0	20	40	59.3	0.000
	Mild	0	0	15	30	0	0	15	30		
	Moderate	0	0	3	6	3	6	6	12		
	Strong	0	0	0	0	9	18	9	18		
ER	**Negative**	1	2	4	8	10	20	15	30	40.18	0.000
	Mild	1	2	13	26	2	4	16	32		
	Moderate	2	4	8	16	0	0	10	20		
	Strong	7	14	2	4	0	0	9	18		
	Total	**11**	**22**	**27**	**54**	**12**	**24**	**50**	**100**		

Table 2. Bcl2, Bax, P53 and ER expression in relation to staging of the tumor.

		S	I	S	II	S	III	S	IV	Total		Chi-square test	
		No	%	No	%	No	%	No	%	No	%	value	P value
Bcl2	**Negative**	1	2	8	16	2	4	2	4	13	26	16.31	0.061
	Mild	5	10	10	20	2	4	1	2	18	36		
	Moderate	3	6	1	2	0	0	2	4	6	12		
	Strong	8	16	5	10	0	0	0	0	13	26		
Bax	**Negative**	1	2	8	16	0	0	2	4	11	22	12.73	0.175
	Mild	4	8	6	12	3	6	0	0	13	26		
	Moderate	9	18	8	16	1	2	2	4	20	40		
	Strong	3	6	2	4	0	0	1	2	6	12		
P53	**Negative**	9	18	8	16	3	6	0	0	20	40	13.38	0.146
	Mild	4	8	8	16	1	2	2	4	15	30		
	Moderate	3	6	3	6	0	0	0	0	6	12		
	Strong	1	2	5	10	0	0	3	6	9	18		
ER	**Negative**	2	4	9	18	0	0	4	8	15	30	16.17	0.062
	Mild	5	10	8	16	2	4	1	2	16	32		
	Moderate	4	8	4	8	2	4	0	0	10	20		
	Strong	6	12	3	6	0	0	0	0	9	18		
	Total	**17**	**34**	**24**	**48**	**4**	**8**	**5**	**10**	**50**	**100**		

Table 3. Immunoreactivity of Bcl2 in relation to ER, P53& Bax of 50 cases of invasive ductal carcinoma.

	ER		P53		Bax	
	Positive	**Negative**	**Positive**	**Negative**	**Positive**	**Negative**
Bcl2 Positive	30(60%)	7 (14%)	19(38%)	18(36%)	33(66%)	4(8%)
Negative	5 (10%)	8(16%)	11(22%)	2(4%)	6(12%)	7 (14%)
Total	35(70%)	15(30%)	30(60%)	20(40%)	39(78%)	11(22%)
P Value*	0.011		0.035		0.003	

*Fisher exact test

Table 4. Immunoreactivity of Bax in relation to Bcl2, ER& P53 of 50 cases of invasive ductal carcinoma.

	Bcl2		P53		ER	
	Positive	**Negative**	**Positive**	**Negative**	**Positive**	**Negative**
Bax Positive	33(66%)	11(12%)	21(42 %)	18 (36%)	31(62%)	8(16%)
Negative	4(8 %)	7 (14%)	9(18%)	2 (4%)	4(8 %)	7(14%)
Total	37(74%)	13(26%)	30(60%)	20 (40%)	(70%)	(30%)
P Value*	0.003		0.163		0.01	

*Fisher exact test

Table 5. Kaplan Meier survival analysis of overall survival.

Variables		**Mean**	**SEM**	**Median**	**Log-rank test**
Total		**47.19**	**3.7**	**40**	
Grade	1& 2	52.3	4.2	48	P= 0.001*
	3	30	5.2	28	
Stage	I&II	51.5	3.7	48	P= 0.06
	III&IV	28.4	2.0	16	
Bax	Negative	35.2	5.9	30	P= 0.048*
	Positive	50.8	4.3	48	
Bcl2	Negative	34	6.95	28	P= 0.01*
	Positive	53	4.3	50	
P53	Negative	54.5	4.9	55	P= 0.49*
	Positive	42.4	5.0	36	
ER	Negative	36.9	6.12	30	P= 0.1
	Positive	51.4	4.4	48	

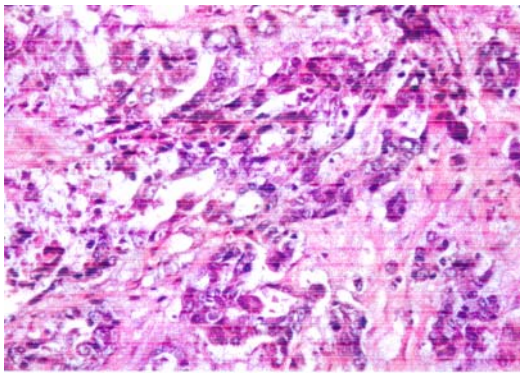

Fig 1. G II infiltrating duct carninoma-Hx & E

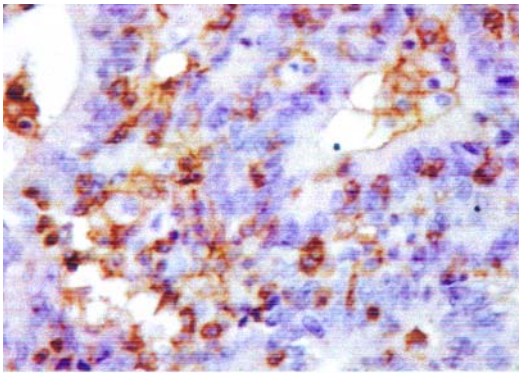

Fig 2. Bcl-2 immunostaning decorates cell border and cytoplasm.

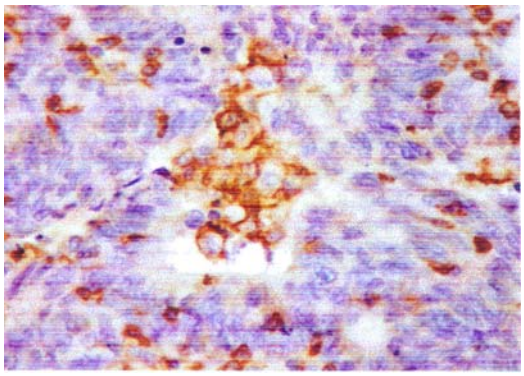

Fig 3. Bcl-2 immunostaning for another case.

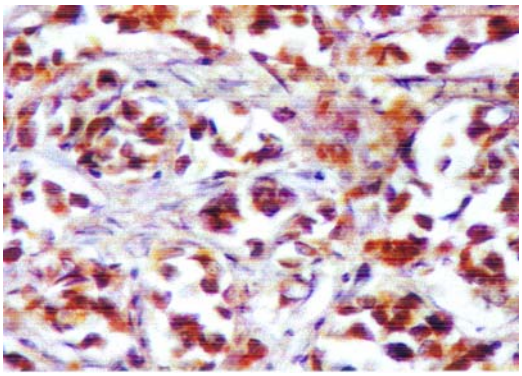

Fig 4. Bax immunostaning evident in the cytoplasm.

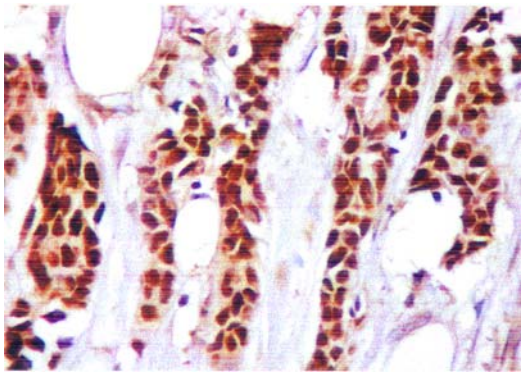

Fig 5. Immunostaning for p53 shows positive nuclear staining.

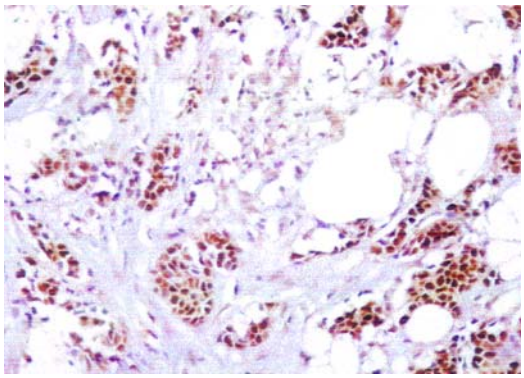

Fig 6. Immunostaning for ER shows positive nuclear staining of tumour cells.

DISCUSSION

Apoptosis programmed cell death is an actively regulated cellular process that leads to the destruction of individual cells. It plays a critical role in a variety of physiological processes during fetal development and in adult life and can be trigerred by several stimuli such as radiation, drugs, toxins. The apoptotic process is controlled by several genes including (P53, Bclxs, Bax and Bak) and represses by (Bcl2, BlxL and MCI-I). The balance between expression of these genes regulated the cell cycle and apoptosis. These balance

is regulated by other stimuli such as P53 proteins or oestrogen receptors in breast carcinomas. Excess of Bcl2 promotes cell survival by inhibiting apoptosis, whereas excess of Bax accelerates cell death.[15]

Normal Bcl-2 protein is usually synthesized in cells undergoing proliferation or in well-differentiated, noncycling cells (permanent cells). Furthermore, normal proliferating cells (ie, intestinal or breast ductal cells) lose their capability of synthesizing Bcl-2 prior to undergoing apoptosis.[16,17] Unlike many other known human oncogenes, bcl-2 exerts its influence by enhancing cell survival rather than stimulation.[18]

The role of p53 in apoptosis has also been demonstrated; p53 gene mutations, inducing loss of p53 function, confers resistance to apoptosis.[19,20] In this scenario, the increase in the number of tumor cells would result from both unregulated proliferation and resistance to cell death. The latter mechanism is reminiscent of the one operating in follicular B-cell lymphomas in which overexpression and activation of Bcl-2 by chromosomal translocations perpetuates to the increase in the number of malignant B lymphocytes.[21] Unlike many other known human oncogenes, bcl-2 exerts its influence by enhancing cell survival rather than stimulation.[18]

In our study, Bcl2 expression displayed a negative relation with increasing histological grades and stages. This result is in agreement with the finding of Leek et al 6 who reported the relation of Bcl-2-negative breast tumors with poor prognosis. Our finding, however, is not coincides with that of Domenico et al.[22] who observed a negative or decreased Bcl-2 immunoreactivity in the low-stage tumors and intensely decorate the tumor cells in the high-stage carcinomas. This incongruence may reflect a difference in stage between the tumors in our study and those examined by Domenico and associates. Also our results is not similar to that of Silvestrini et al23 who, in their study of 283 breast carcinomas, found an unfavorable predictive value of Bcl-2 expression that was mainly dependent on p53 expression.

Interestingly, we found an inverse relationship between p53 and Bcl-2 immunoreactivity. This finding may reflect a bcl-2 gene downregulation of a mutated and/or wild-type p53 protein, a phenomenon also observed in vitro, in human breast carcinoma cell lines.[24] It is possible that a dual mechanism may be active at different times during the carcinogenic process, in which both Bcl-2 and p53 interfere with each other's regulatory processes. This hypothesis is supported by the recent finding that overexpressed Bcl-2 may suppress p21 (Waf1) expression independently of p53 and may alter cell cycle regulation.25

Reports have shown a strong negative relationship between estrogen receptors and p53 expression[26,27] and between progesterone receptors and Bcl-2 expression,[28] as well as a positive association between Bcl-2 expression and estrogen-receptor-positive tumors.[23] In this small study, relation between the expression of estrogen receptors and Bcl-2 expression were also detected.

In accordance with Rehman et al.,[29] who studied Bax, Bcl2, ER and P53 in 58 invasive cancers revealed that Bax expression Bax was found in 67% of cases and was associated to increasing histological grade of IDC. It did not associate to ER or p53 but positive relation was seen with Bcl2. In our study, Bax expression was found in 78% of cases and similar to Rehman et al., it was positively associated to increasing histological grade of IDC and no relation between Bax and ER was found but positive relation was seen with Bcl2. In another study by Craig et al.,[10] Bax was studied in IDC and found to be expressed in 75% of cases.It did not associate to tumour grade, ER, P53 or Bcl2.

The prognostic significance of Bcl-2 may be enhanced by inclusion of Bax data in patients with p53-immunopositive adenocarcinoma of the breast, at least for patients with node-negative disease. These data suggest that, despite the ability of p53 to bind directly to the Bax gene promoter, the regulation of Bax in human breast cancers does not necessarily associate with p53 status, implying that regulation of this pro-apoptotic gene in these tumors is complex and probably influenced by several factors.[30,31]

In summary, the regulation of apoptosis is important in invasive ductal carcinomas. Bcl2 is a good prognostic marker and its overexpression in some tumors may extend survival of the tumor cells. In other tumors, an altered p53 oncoprotein may be unable to suppress growth and proliferation of neoplastic cells but may be capable of downregulating Bcl-2 expression. Our study indicates that p53 and Bcl-2 protein expression may be important in the progression of breast carcinomas. We also noted a significant association between Bcl-2 expression, estrogen receptors. The regulation of Bax is complex and no relation between P53 and Bax which could be explained by the mutation or inactivation of P53, being unable to promote Bax gene expression.

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