Expression of *bcl-2* Protein Predicts Efficacy of Adjuvant Treatments in Operable Node-positive Breast Cancer[1]

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ABSTRACT

The proto-oncogene *bcl-2* encodes a protein that inhibits apoptosis, a common mechanism of cell death caused by hormone and chemotherapy.

We have analyzed *bcl-2* protein expression by immunocytochemistry in primary node-positive breast cancers in two groups of patients (for a total of 180 cases). One group received adjuvant hormone therapy, the other chemotherapy (cyclophosphamide, methotrexate, and fluorouracil), and both groups were followed for a median time of 63 months. We compared our findings with conventional clinicopathological indicators [menopausal status, number of axillary nodes, histological grade, tumor size and type, estrogen receptor (ER), and progesterone receptor] and with p53 protein expression.

***bcl-2* protein was present in 65% of the carcinomas (117/180) and it was significantly associated with ER and progesterone receptor and inversely associated with p53 in both the groups of patients treated with adjuvant chemotherapy and tamoxifen. In patients treated either with adjuvant chemotherapy or tamoxifen, relapse-free survival at 5 years was significantly better among patients with *bcl-2*-positive tumors than in those with *bcl-2* negative ones ($P = 0.05$ and 0.02, respectively). As far as overall survival is concerned, patients with *bcl-2*-positive tumors had a significantly better outcome in the group treated with adjuvant chemotherapy ($P = 0.03$).**

Multivariate analyses were performed for the two treatment groups. In the group treated with tamoxifen, lack of expression of ER and of *bcl-2* was the only significant and independent predictor for poor relapse-free survival ($P < 0.01$). A number of nodes above 3 was the only significant and independent predictor for poor overall survival ($P < 0.01$). In the cyclophosphamide-methotrexate-fluorouracil-treated group, *bcl-2* absence was significant for poor overall survival ($P = 0.02$) as well as a number of nodes above 3 ($P = 0.04$) and a tumor size above 2 cm ($P = 0.05$). For poor relapse-free survival only a number of nodes above 3 ($P < 0.01$) and progesterone negativity ($P = 0.02$) were significant and independent predictors of a higher probability of relapse.

Thus, in contrast to *in vitro* data on drug resistance, *bcl-2* expression was associated with better outcomes in patients treated with hormone and chemotherapy.

Overall, these results suggest that expression of *bcl-2* protein and the number of metastatic lymph nodes are independent features predictive of clinical outcome in patients with node-positive breast cancer, irrespective of the type of adjuvant treatment. The determination of *bcl-2* protein may prove to be a useful tool to distinguish patients for whom conventional forms of adjuvant therapy are beneficial from those with *bcl-2* negative and ER-negative tumors for whom novel therapeutic strategies are needed.

INTRODUCTION

The recent meta-analysis on the long-term efficacy of systemic adjuvant therapy for patients with operable breast carcinoma confirmed the beneficial effect of both adjuvant combination chemotherapy and hormone therapy (1). However, because the magnitude of the effect on treated patients is a survival enhancement of about 20%, the results cannot be considered optimal.

By selection of those patients at high risk, who may benefit most from adjuvant therapy and excluding those with the best prognosis, a better use of resources and better patient management may be possible (2). Markers predictive of responsiveness to chemotherapy or hormone therapy would be helpful in choosing optimum treatments for high-risk patients. Those likely to be resistant to both may be candidates for novel therapeutic strategies. Lymph node status (3) and ER[3] expression (4), the most widely used tools in making decisions to identify those patients eligible for adjuvant treatments, present some limits. Node status *per se* is not a useful predictive marker of response to therapy and, thus, it is of no help to the clinician in identifying the optimal treatment (chemotherapy *versus* hormone therapy or

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[3] The abbreviations used are: ER, estrogen receptor; PgR, progesterone receptor; RFS, relapse-free survival; CMF, cyclophosphamide-methotrexate-fluorouracil; OS; overall survival; OR, odds ratio; CI, confidence interval.

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both) for each patient (2). Moreover, negative node status lacks an absolute prognostic value because about 30% of the node-negative breast cancer patients are at risk of recurrence after adequate local therapy (5) and may benefit from adjuvant therapy (1). Conversely, the determination of ER is of predictive value for response to adjuvant hormone therapy but has little prognostic value (2). We also need to identify indicators of response to a particular form of adjuvant therapy. It seems possible to predict response to adjuvant tamoxifen therapy by ER (1), PgR (6), and other markers (7–9). Although preliminary reports have shown a relationship between response to some forms of chemotherapy and lack of ER (10), cell proliferation (11), heat shock proteins (12), c-*erb*B-2 expression (13), or P-glycoprotein expression (14), currently there is no reliable factor to predict response to adjuvant chemotherapy (2).

In the search of novel potentially useful prognostic or predictive markers the expression of *bcl-2* protein is of particular interest. *bcl-2* protooncogene was first described as a result of the chromosomal translocation [t (14;18)] seen in B cell lymphoma lines (15). It codes for an integral inner mitochondrial membrane protein of 25 kDa (16), although there are multiple intracellular sites of expression (17). This protein protects cells from programed cell death or apoptosis in several experimental models (18, 19) and has an oncogenic effect because of a decreased programed cell loss (20). Inappropriate *bcl-2* protein expression leads to neoplastic growth at a rate slower than that induced by other oncogenes such as signaling transducers involved in tumor cell proliferation (21). Recent experimental studies have shown that many anticancer agents including cytotoxic chemotherapeutics (*e.g.*, topoisomerase inhibitors, DNA-reactive drugs such as cisplatin and antimetabolites; Ref. 22), hormonal agents such as glucocorticoids (22), and antiestrogens (23) act by inducing tumor cell death with the characteristics of apoptosis in cultured cells and *bcl-2* may confer cytotoxic drug resistance (24).

Pezzella *et al.* (25) have documented the clinical importance of the expression of *bcl-2* protein in non-small cell lung cancer patients. Little information on the expression of *bcl-2* is available in human breast cancer; however, at least four groups (26–30) reported that up to 80% of human breast invasive cancers express *bcl-2* protein and that this is closely related to ER and PgR (26, 27, 29, 30). *bcl-2* was inversely related to epidermal growth factor receptor (26), c-*erb*B-2 protein (28, 30), and p53 protein positivity (26, 27, 29). Thus, loss of *bcl-2* expression seems to be associated with a range of markers of poor prognosis in breast cancer.

Another oncogene involved in both apoptosis and drug resistance is *p53* (31–33). Mutations in this gene are the most frequent genetic change in human breast cancer (34). Several studies have reported that *p53* overexpression is a significant and independent prognosticator in early-stage breast cancer (35, 36). Mutant forms of p53 stimulate the expression of the *MDR-1* gene (*i.e.*, multidrug resistance to chemotherapy) (32, 33). To establish whether *bcl-2* expression is related to clinical outcome of patients treated with adjuvant therapy, we evaluated two groups of node-positive breast cancer patients (180 patients) for whom detailed information on tumor characteristics and long-term follow-up was available. One group received adjuvant tamoxifen, the other adjuvant chemotherapy. Because the treatments function in different ways and the selection criteria for these adjuvant treatments result in different types of breast cancer in each group, it was appropriate to analyze each separately.

PATIENTS AND METHODS

Patients. One hundred eighty consecutive, unselected, patients with node-positive breast carcinoma who had undergone breast cancer surgery at the St. Bortolo Medical Centre of Vicenza, or at the St. Chiara Medical Centre of Trento from January 1986 to December 1988 were studied. Criteria of elegibility were: histological diagnosis of invasive breast cancer, stages T_1-T_{3a} and presence of histologicaly confirmed metastases in axillary lymph nodes (stages N_{1-2}), with at least levels I and II cleared, no distant metastasis (Mo), unilateral tumor, and no other previous or concomitant invasive tumor. Local surgical treatments were modified radical mastectomy in 103 patients or quadrantectomy with axillary dissection as advocated by Veronesi *et al.* (37) in 77 patients. In all of the patients treated with quadrantectomy a 5- to 6-week course of radiation therapy was given within 6 weeks from surgery. Quadrantectomy was performed as an alternative to modified radical mastectomy in those patients with primary tumors less than 3 cm in diameter.

Adjuvant Treatments. All patients received, within 4 weeks from surgery, adjuvant systemic therapy following these criteria: CMF chemotherapy given by short i.v. infusion (in the sequence) at the doses of 600 mg/m^2, 40 mg/m^2, and 600 mg/m^2, respectively, for 8 cycles every 21 days to premenopausal or perimenopausal patients, irrespective of the ER status (72 patients) and to postmenopausal patients less than 65 years old with more than 3 axillary nodes involved (27 patients) (total treated with CMF, 99 patients). Postmenopausal patients less than 65 years old with less than 3 axillary nodes involved and all those more than 65 years old received tamoxifen 20 mg *b.i.d.* daily for at least 3 years (total treated with tamoxifen, 81 patients).

Table 1 shows the main characteristics of the series of patients studied by the adjuvant treatment.

Follow-up. Physical examination was performed monthly during adjuvant chemotherapy and then every 4 months in all women. Radiographical studies including chest roentgenogram, mammography, and liver echotomography were carried out every 6 months, or earlier whenever clinically indicated. Hematological tests, including 12 channel biochemical profile and complete blood cell counts, were repeated every 6 months. RFS and OS were calculated as the period from surgery until the date of the first recurrence (RFS) or death (OS).

Primary treatment failure was defined as the first documented evidence of new disease manifestation(s) in locoregional area(s), distant site(s), contralateral breast, or a combination of the above. Any new disease involvement was accurately assessed by clinical, radiological, and, whenever feasible, histological examination of the site(s) of first relapse.

Histopathological Studies. Surgical specimens were routinely formalin fixed and paraffin embedded. Tumors were classified according to the National Surgical Adjuvant Breast Project (38): 149 (82.5%) were infiltrating ductal carcinomas, 23 (13%) infiltrating lobular carcinomas, and 8 (4.5%) other

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Table 1 Patient clinicopathological characteristics stratified by adjuvant treatment

Feature	Adjuvant tamoxifen (%)	Adjuvant CMF (%)	Total no.
Adjuvant therapy	81 (45)	99 (55)	180
Median age, yr (range)	64 (47–83)	48 (33–65)	
Menopausal status			
Premenopausal	0 (0)	56 (56.5)	56
Perimenopausal	0 (0)	16 (16)	16
Postmenopausal	81 (100)	27 (27.5)	108
Histotype			
Ductal infiltrating	68 (84)	81 (82)	149
Lobular infiltrating and others	13 (16)	18 (18)	31
Pathological tumor size			
pT1	39 (48)	51 (51.5)	90
pT2-3	42 (52)	48 (48.5)	90
Histological grading			
I–II	34 (42)	37 (37)	71
III	47 (58)	62 (63)	109
No. of involved nodes, median (range)	3 (1–30)	3 (1–25)	
<3	40 (49)	47 (47.5)	87
≥3	41 (51)	52 (52.5)	93
bcl-2 expression			
Negative	25 (31)	38 (38)	63
Positive	56 (69)	61 (62)	117
p53 expression			
Negative	65 (80)	78 (79)	143
Positive	16 (20)	17 (17)	33
ND[a]	0 (0)	4 (4)	4
ER expression			
Negative	37 (45.5)	66 (66.5)	103
Positive	44 (54.5)	32 (32.5)	76
ND	0 (0)	1 (1)	1
PgR expression			
Negative	53 (65)	71 (71.5)	124
Positive	28 (35)	27 (27.5)	55
ND	0 (0)	1 (1)	1

[a] ND, not done.

types of infiltrating carcinomas. Grading of the neoplasms was performed using the modified Bloom and Richardson's method according to Elston and Ellis (39).

All identifiable lymph nodes in the I-II (at least) axillary levels were histologically examined. The median number of cleared axillary lymph nodes was 13 (range, 6–30) and the median number of those metastatic was 3 (range, 1–30) in both treatment groups.

Immunohistochemical Studies. *bcl-2* immunoreactivity was assessed on paraffin-embedded sections using the mAb 124 (DAKO, Glostrup, Denmark) as previously described (27), with a microwave antigen-retrieval system. Slides were incubated with the antibody at 1:200 dilution for 1 h at room temperature, and processed using the highly sensitive streptavidin-biotin immunohistochemical method. Negative controls were obtained omitting the primary antibody; lymphocytes were used as internal positive controls for *bcl-2* expression.

bcl-2 expression was categorized as follows: negative (−) if no staining was seen in tumor cells or if only a weak-positive and heterogeneous staining was observed in less than 25% of the tumor cells, with an intensity inferior to that of lymphocytes, or positive (+) if more than 25% of the cells were stained with an intensity similar to or greater than lymphocytes. Further subcategorization into four levels of intensity of staining did not improve assessment.

p53 protein immunoreactivity was evaluated on paraffin-embedded sections using mAb anti-p53 DO7 (Novocastra Lab, Newcastle Upon Tyne, United Kingdom) (40). Sections were incubated for 2 h at 4°C with the primary antibody at 1:200 dilution. Biotinylated horse anti-mouse IgG at 1:200 dilution and avidin-biotin-peroxidase complex at 1:100 dilution were added in sequence (Vectastatin ABC kit; Vector Laboratories, Inc., Burlingame, CA). Positive controls for p53 were sections of breast and larynx carcinomas known to express *p53* and/or to bear *p53* gene mutations. Negative controls consisted of omission of the primary antibody.

Only nuclear p53 staining was evaluated, counting 1000–2000 positive nuclei/tumor. The marker was categorized as follows: positive (+) if there was more than 20% of nuclei labeled and anything less as negative (−). Further subcategorization into four levels of intensity of staining did not improve assessment.

ERs and PgRs were assessed in paraffin sections using the H-222 and the KD-68 rat mAbs, respectively (Abbott Laboratories, North Chicago, IL). Positive and negative controls were run in parallel with the sections under investigation. The receptors were classified as follows: negative (−) if <10% of the cells showed nuclear reactivity and positive (+) if ≥10% nuclei were immunostained. Further subcategorization into three levels of intensity of staining did not improve assessment.

All pathobiological features were evaluated separately by two observers without knowledge of clinical outcome of the patients, in a blind manner.

Statistical Analysis. Statistical analysis was performed separately for each adjuvant treatment group. The association of *bcl-2* protein expression (categorized as positive/negative) with the other variables determined was evaluated using a logistic regression model. In this model each regression coefficient is the logarithm of OR (odds is the probability of *bcl-2* positivity over the probability of *bcl-2* negativity). Under the null hypothesis of no association between *bcl-2* and another variable, OR is expected to be 1.0.

The patterns of OS and RFS were estimated by means of the product limit method (Kaplan-Meier) on the basis of a 6-year follow-up period.

The role of each of the prognostic variables (univariate analysis) and their joint effect (multivariate analysis) on OS and RFS were evaluated using a log-logistic regression model where each regression coefficient is the log of OR and is constant in time, as reported previously (41).

We adopted an accelerated failure-time model as suggested by Gore *et al.* (42) which is particularly suitable for breast cancer. They demonstrated that in patients with early-stage breast cancer the hazard function of OS is typically nonmonotone and nonproportional and that the time to peak hazard is in general around the third year. We observed this pattern also for RFS in different case series (29, 41). The log-logistic regression model accommodates the variable time to peak hazard and the asymptotic ratio of hazards is 1. It is an accelerated failure-time model and has the property that the ratio of the odds on death before t is constant between prognostic groups. Bennet (43)

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showed how graphical data should conform to the log-logistic model, *i.e.*, for each variable the plots of log(odds) *versus* log(t) give reasonably parallel straight lines.

For OS odds are the probability of dying over the probability of surviving, for RFS odds are the probability of relapsing over the probability of remaining disease free. We were interested in the comparison of multivariate analysis results between the two treatment groups. Therefore, for each group we adopted an initial model containing the same variables (all those with OR significantly different from 1.0 in at least one of the univariate analysis performed on each treatment group) and the interaction terms considered biologicaly relevant (ER and *bcl-2*, PgR and *bcl-2*, p53 and *bcl-2*). A final more parsimonious model was then obtained using a backward selection procedure.

In all instances the OR value refers to the category at higher risk in the comparison for each variable.

The approach suggested by Gail and Simon (44) was applied to the interactions that resulted statistically significant in order to investigate the presence of a quantitative or qualitative interaction.

Statistical analysis was performed by the Statistical Analysis System package (SAS Institute, Cary, NC).

RESULTS

Clinical Outcome of the Patients

At a median follow-up of 63 (range, 8–90) months, the probability of RFS and OS in the total patient population (*i.e.*, both series) was 59% and 69%, respectively. During the period of observation 76 patients had recurrences and 58 patients died (46 and 30 patients and 30 and 28 patients in the groups treated with adjuvant CMF chemotherapy and adjuvant tamoxifen therapy, respectively). In the survival analysis we included all of the causes of death. Six patients (four in the group treated with adjuvant endocrine therapy and two in the group treated with chemotherapy) died for other causes (two cardiovascular diseases, one lung cancer, one car accident, one ictus cerebri, and one for diabetes complications). The probability of RFS and OS at 63 months was similar for patients treated with adjuvant CMF chemotherapy and those treated with adjuvant tamoxifen (χ^2 = 0.18, P = 0.66 and χ^2 = 0.51, P = 0.47, respectively).

bcl-2 Protein Expression

Of the 180 tumors analyzed, 117 (65%) were found to have cytoplasmic staining for the *bcl-2* protein. The intensity of staining was heterogeneous. Fig. 1*A* shows a carcinoma with strong and diffuse positivity, whereas Fig. 1*B* shows a negative case.

Normal ducts and lobules adjacent to the tumor showed heterogeneous staining for *bcl-2*. Myoepithelial cells and fibroblasts were negative, whereas intratumoral lymphocytes were always positive with a high intensity of staining. As reported in Table 2, *bcl-2* protein in both the adjuvant treatment groups was significantly associated only with ER and PgR expression. ORs for ER and PgR were 2.96 and 5.91, respectively, in the group treated with tamoxifen and 2.97 and 2.71, respectively, in the group treated with chemotherapy. In both groups *bcl-2* was inversely associated with p53 expression (ORs of 0.25 and 0.33, for the group treated with tamoxifen and chemotherapy, respectively).

bcl-2 was not significantly associated with the number of axillary nodes involved.

Other Markers

The distribution of the clinicopathological features is shown in Table 1. In our series 76 tumors were ER positive and 55 were PgR positive. Fifty-two percent (90/176) of the tumors were positively stained by the anti-p53 antibody we used, but only 33 tumors (18.5%) had more than 20% of cells labeled and were considered p53 positive for the purpose of this study.

Survival Results

Survival of Patients Treated with Adjuvant Tamoxifen. In univariate analysis *bcl-2*-positivity was significantly predictive for good RFS (P = 0.02), but not for OS (P = 0.11) (Fig. 2). Besides *bcl-2* expression, p53 negativity and ER positivity were also significantly predictive for good RFS. For OS, those patients with a number of involved nodes less than 3, with p53-negative and with ER-positive tumors had significant better survival experience.

In multivariate analysis (final model) only the interaction between *bcl-2* and ER was statistically significant. Among the patients with ER-positive tumors the odds of relapse of those *bcl-2*-positive were slightly higher when compared to the *bcl-2*-negative ones (OR = 0.27, CI = 0.02–2.76, P = 0.26). On the contrary, among the patients with ER-negative tumors the odds of relapse of those *bcl-2*-positive were significantly lower than those of the *bcl-2*-negative ones (OR = 5.70, CI = 1.71–24.44, $P < 0.01$) (Table 3 and Fig. 3*A*). These findings suggest that *bcl-2* added significant predictive information only in the patients with ER-negative tumors.

As far as OS is concerned, the final model of multivariate analysis showed that among the patients with ER-positive tumors the OR for *bcl-2* positive *versus* negative was 1.16 (CI = 0.16–8.12, P = 0.87) whereas for those ER-negative tumors the corresponding OR for *bcl-2* positive *versus* negative was 1.78 (CI = 0.49–6.89, P = 0.36). The interaction between ER and *bcl-2* was not statistically significant, but we retained it in the model in order to be able to compare the results between RFS and OS (Fig. 3*B*). The only variable which retained a significant and independent predictive value for OS was the number of involved lymph nodes ($P < 0.01$) (Table 3).

Survival of Patients Treated with Adjuvant Chemotherapy. In this group of patients *bcl-2* positivity was significantly predictive for better RFS (P = 0.05) and OS (P = 0.03) (Fig. 4). In univariate analysis PgR positivity (P = 0.02), a tumor size <2 cm (P = 0.03), and a number of lymph nodes less than 3 ($P < 0.01$) were also predictive of better RFS. Besides *bcl-2* positivity, also p53 negativity (P = 0.01), a tumor size <2 cm (P = 0.02) and a number of lymph nodes less than 3 (P = 0.02) were significantly predictive for better OS.

In the multivariate analysis all interaction terms were not statistically significant, hence the results reported in Table 4 concern only the main effects of the following variables: number of involved nodes, tumor size, p53, ER, PgR, and *bcl-2*. In the final model PgR positivity (P = 0.02) and a number of lymph nodes less than 3 ($P < 0.01$) were significant and independent factors for better RFS. *bcl-2* positivity (P = 0.02),

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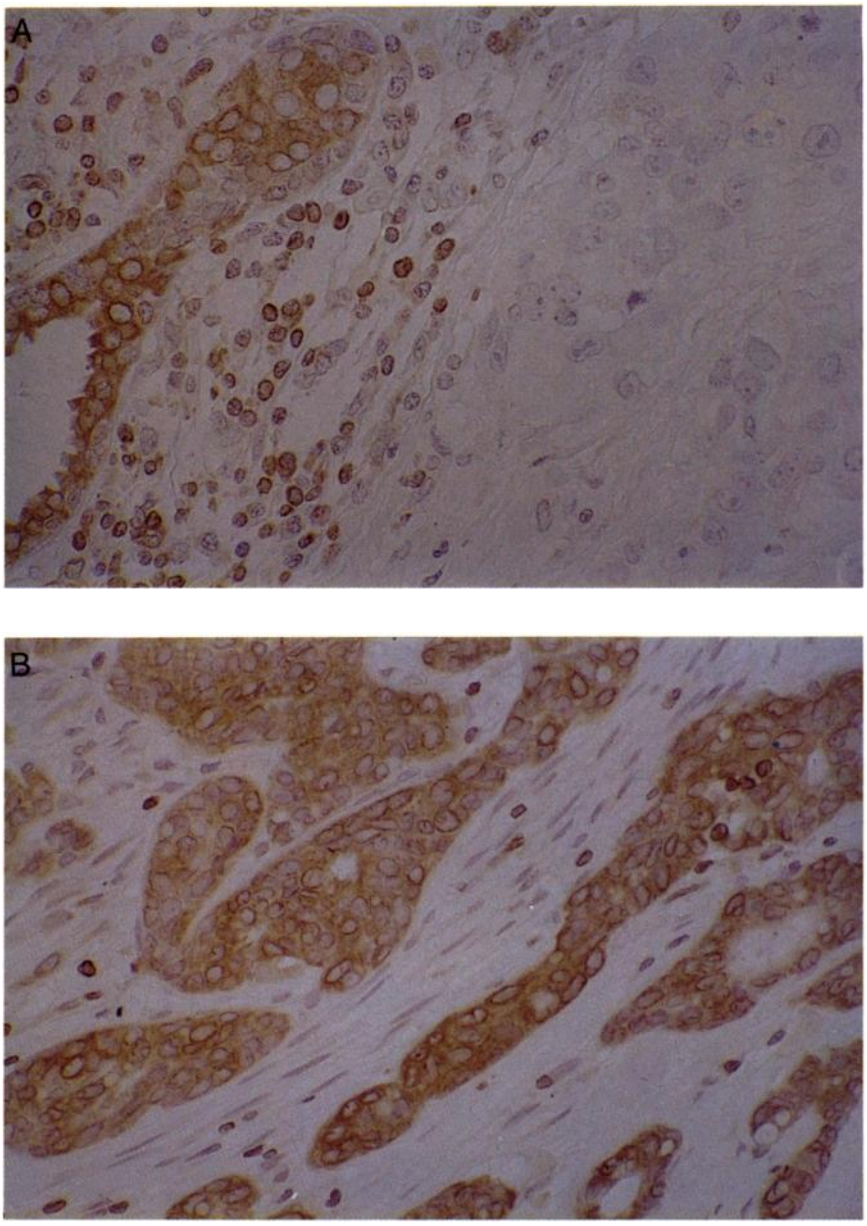

Fig. 1 *A*, *bcl-2* immunostaining in an infiltrating ductal carcinoma showing no reaction in the neoplastic cells. Lymphocytes and normal mammary ductal cells show intense staining. *B*, photograph showing a node-positive breast infiltrating ductal carcinoma with strong and intense cytoplasmic staining for the mAb 124 to *bcl-2*. *A* and *B*, × 400.

Table 2 Expression of the *bcl-2* protein by hormone receptors and p53 expression

	Adjuvant tamoxifen						Adjuvant CMF					
Variable	*bcl-2*$^-$ *n* (%)	*bcl-2*$^+$ *n* (%)	OR	95% CI[a]	χ^2	*P*	*bcl-2*$^-$ *n* (%)	*bcl-2*$^+$ *n* (%)	OR	95% CI	χ^{2b}	*P*
ER$^+$	9 (11)	35 (43)	2.96	1.11–7.89	4.72	0.03	7 (7)	25 (25.5)	2.97	1.13–7.83	4.87	0.03
ER$^-$	16 (20)	21 (26)					30 (30.5)	36 (37)				
PgR$^+$	3 (4)	25 (30)	5.91	1.58–22.05	7.00	0.008	6 (6)	21 (21.5)	2.71	0.97–7.53	3.66	0.05
PgR$^-$	22 (27.5)	31 (38.5)					31 (31.5)	40 (41)				
p53$^+$	9 (11)	7 (8.5)	0.25	0.08–0.79	5.57	0.02	10 (10.5)	7 (7)	0.33	0.11–0.97	4.06	0.04
p53$^-$	16 (20)	49 (60.5)					25 (26)	53 (56)				

[a] CI, 95% confidence intervals.
[b] Wald statistic.

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Table 3 Univariate and multivariate analyses of RFS and OS for patients treated with adjuvant tamoxifen (log-logistic regression model)[a]

	RFS				OS			
			Wald statistic				Wald statistic	
Variable Category	OR	CI	χ^2	*P*	OR	CI	χ^2	*P*
A.				Univariate analysis				
bcl-2$^+$ *versus* $-$[b]	3.03	1.20–9.09	5.41	0.02	2.22	0.84–6.25	2.68	0.11
p53$^-$ *versus* $+$[b]	3.88	1.46–12.14	7.17	<0.01	2.66	0.96–7.96	3.62	0.05
ER$^+$ *versus* $-$[b]	5.69	2.27–17.01	12.68	<0.01	3.77	1.45–10.96	6.97	<0.01
Lymph nodes <3 *versus* ≥3[b]					4.32	1.63–13.07	8.10	<0.01
B.				Multivariate analysis: initial model				
ER$^-$/*bcl-2*$^+$ *versus* ER$^-$/*bcl-2*$^-$	3.84	1.07–16.55	4.30	0.03	1.45	0.37–5.91	0.32	0.56
ER$^+$/*bcl-2*$^+$ *versus* ER$^+$/*bcl-2*$^-$	0.34	0.02–3.88	0.79	0.37	0.99	0.13–7.16	0.00	0.99
p53$^-$ *versus* $+$[b]	2.53	0.82–8.60	2.67	0.10	2.06	0.66–6.84	1.62	0.20
PgR$^+$ *versus* $-$[b]	0.45	0.11–1.76	1.34	0.24				
Lymph nodes <3 *versus* ≥3[b]	2.23	0.82–6.83	2.54	0.11	4.73	1.62–15.97	7.63	<0.01
Tumor pT1 *versus* size pT2-3[b]	1.53	0.56–4.39	0.73	0.39	1.07	0.38–3.09	0.02	0.81
C.				Multivariate analysis: final model				
ER$^-$/*bcl-2*$^+$ *versus* ER$^-$/*bcl-2*$^-$[b]	5.70	1.71–24.44	7.92	<0.01	1.78	0.49–6.88	0.82	0.36
ER$^+$/*bcl-2*$^+$ *versus* ER$^+$/*bcl-2*$^-$[b]	0.27	0.02–2.76	1.26	0.26	1.16	0.16–8.12	0.02	0.87
Lymph nodes <3 *versus* ≥3[b]				4.41	1.56–14.27	7.39	<0.01	

[a] Other variables not significantly associated with RFS were: PgR$^+$ *versus* $-$, $P = 0.37$; grading I–II *versus* III, $P = 0.31$; lymph nodes <3 *versus* ≥3, $P = 0.12$; tumor size pT1 *versus* pT2-3, $P = 0.46$; histotype ductal *versus* others, $P = 0.75$; and menopausal status premenopausal-perimenopausal *versus* postmenopausal, $P = 0.28$; or with OS were: PGR$^+$ *versus* $-$, $P = 0.15$; grading I–II *versus* III, $P = 0.82$; tumor size PT1 *versus* pT2-3, P - 0.94; histotype ductal *versus* others, $P = 0.53$; and menopausal status premenopausal-perimenopausal *versus* postmenopausal, $P = 0.96$.

[b] High-risk category in the comparison for each variable.

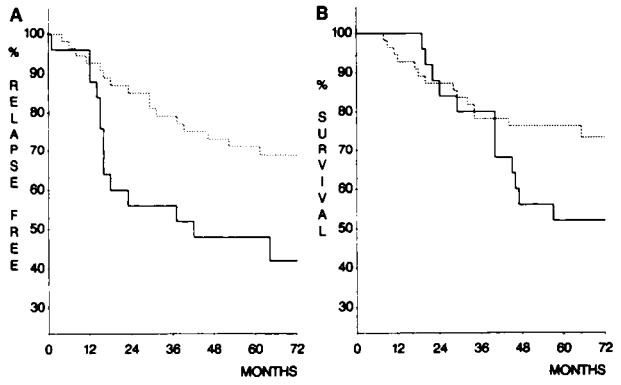

Fig. 2 *A*, 6-year RFS in the group of patients treated with adjuvant tamoxifen with *bcl-2*-positive tumors (56 patients) (····) and with *bcl-2*-negative tumors (25 patients) (—). *B*, 6-year OS in patients with *bcl-2*-positive tumors (····) and with *bcl-2*-negative tumors (—).

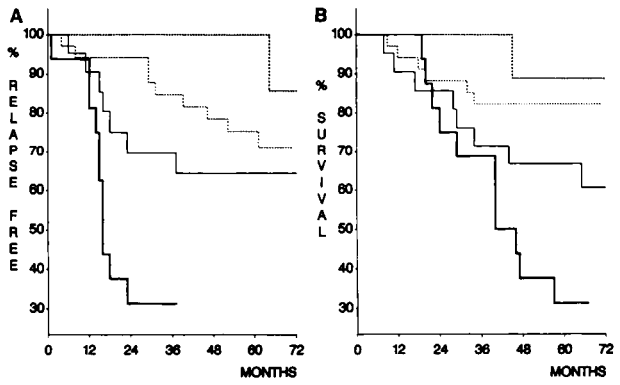

Fig. 3 *A*, 6-year RFS of the group of patients treated with adjuvant tamoxifen. Patients with *bcl-2*-negative and estrogen-negative tumors (16 patients) (—), with *bcl-2*-negative and estrogen-positive tumors (9 patients) (····), with *bcl-2*-positive and estrogen-negative tumors (21 patients) (—), and with *bcl-2*-positive and estrogen-positive tumors (35 patients) (····). *B*, 6-year OS in the group of patients treated with adjuvant tamoxifen. Patients with *bcl-2*-negative and estrogen-negative tumors (—), with *bcl-2*-negative and estrogen-positive tumors (····), with *bcl-2*-positive and estrogen-negative tumors (—), and with *bcl-2*-positive and estrogen-positive tumors (····).

a tumor size less than 2 cm ($P = 0.05$), and a number of lymph nodes less than 3 ($P = 0.04$) were predictive for better OS. p53 expression failed to retain significance in the multivariate analysis.

A common finding in our multivariate analyses is that *bcl-2* expression adds predictive information to the number of metastatic lymph nodes on the clinical outcome of breast cancer patients with node-positive tumors, irrespective of the type of adjuvant treatment given (chemotherapy or hormone therapy).

Discussion

It has been proposed that tumor growth is the result of a balance between cell proliferation and programmed cell death (or apoptosis) (45). Pharmacological manipulation of apoptosis may offer novel therapeutic strategies for the treatment of cancer (46). *bcl-2* protooncogene suppresses apoptosis (18, 19) and because it also protects against apoptosis induced by many anticancer agents, we hypothesized that it would be useful to select patients whose tumors are likely to be resistant to conventional therapy and hence candidates for more intensive or

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Table 4 Univariate and multivariate analyses of RFS and OS for patients treated with adjuvant chemotherapy (log-logistic model)[a]

	RFS				OS			
			Wald statistic				Wald statistic	
Variable Category	OR	CI	χ^2	*P*	OR	CI	χ^2	*P*
A.				Univariate analysis				
bcl-2$^+$ *versus* $-$[b]	2.22	1.01–5.00	3.90	0.05	2.77	1.14–7.14	4.95	0.03
PgR$^+$ *versus* $-$[b]	3.19	1.21–8.97	5.47	0.02				
Lymph nodes <3 *versus* ≥3[b]	4.12	1.85–10.09	11.44	<0.01	3.02	1.20–8.17	5.40	0.02
Tumor size pT1 *versus* pT2-3[b]	2.32	1.08–5.32	4.62	0.03	3.05	1.24–8.16	5.75	0.02
p53$^-$ *versus* $+$[b]					3.82	1.39–12.34	6.55	0.01
B.				Multivariate analysis: initial model				
bcl-2$^+$ *versus* $-$[b]	1.96	0.78–5.26	2.10	0.15	3.44	1.28–11.11	5.79	0.02
p53$^-$ *versus* $+$[b]	1.71	0.62–4.94	1.12	0.29	2.87	0.99–9.67	3.81	0.05
PgR$^+$ *versus* $-$[b]	3.59	1.26–11.24	5.69	0.02				
ER$^+$ *versus* $-$[b]	1.31	0.53–3.44	0.35	0.55	0.69	0.23–1.98	0.50	0.48
Lymph nodes <3 *versus* ≥3[b]	4.04	1.71–10.57	9.74	<0.01	2.51	0.92–7.27	3.32	0.07
Tumor size pT1 *versus* pT2-3[b]	1.83	0.79–4.46	2.04	0.15	2.49	0.94–7.22	3.42	0.06
C.				Multivariate analysis: final model				
bcl-2$^+$ *versus* $-$[b]	1.85	0.80–4.54	2.10	0.15	2.94	1.19–8.33	5.31	0.02
Lymph nodes <3 *versus* ≥3[b]	4.74	2.07–12.11	12.90	<0.01	2.72	1.04–7.64	4.20	0.04
PgR$^+$ *versus* $-$[b]	3.49	1.26–10.43	5.76	0.02				
Tumor size pT1 *versus* pT2-3					2.57	1.01–7.06	3.92	0.05

[a] Other variables not significantly associated with RFS were: p53$^-$ *versus* $+$, $P = 0.10$; ER$^+$ *versus* $-$, $P = 0.71$; grading I–II *versus* III, $P = 0.31$; histotype ductal *versus* others, $P = 0.76$; and menopausal status premenopausal-perimenopausal *versus* postmenopausal, $P = 0.11$ or with OS were: ER$^+$ *versus* $-$, $P = 0.99$; PgR$^+$ *versus* $-$, $P = 0.13$; grading I–II *versus* III, $P = 0.11$; histotype ductal *versus* others, $P = 0.37$; and menopausal status premenopausal-perimenopausal *versus* postmenopausal, $P = 0.51$.

[b] High-risk category in the comparison for each variable.

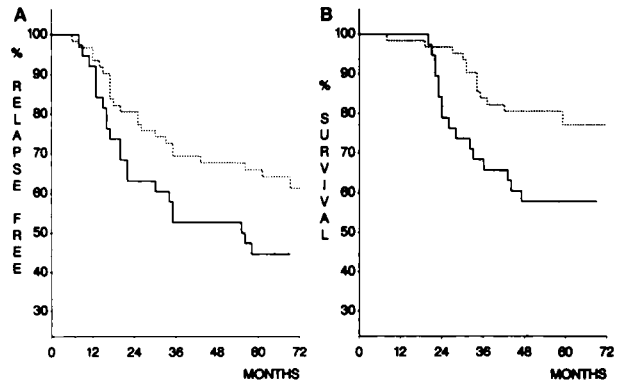

Fig. 4 *A*, 6-year RFS in the group of patients treated with adjuvant chemotherapy with *bcl-2*-positive tumors (61 patients) (····) and with *bcl-2*-negative tumors (38 patients) (—). *B*, 6-year OS in the group of patients treated with adjuvant chemotherapy with *bcl-2*-positive tumors (····) and with *bcl-2*-negative tumors (—).

experimental therapeutic approaches. A study performed on leukemias has shown that elevated levels of expression of regulated *bcl-2* was associated with resistance to various chemotherapeutic agents (47).

In the present study immunostaining of tumors from patients operated on for node-positive breast cancer and treated with adjuvant therapy revealed a high frequency of *bcl-2* expression in accordance with other previous studies (26–30). We confirmed a strong association of ER and PgR with *bcl-2* expression (26, 27, 29, 30). *bcl-2* is present in normal breast and may thus have a role in normal cyclical breast development. It is likely to be under the control of an estrogen-dependent transcriptional pathway. We also observed a significant inverse association between *bcl-2* expression and p53 positivity, in accordance with the results reported in other series (26, 27, 29).

In our study, multivariate analyses performed separately in the group treated with tamoxifen from those treated with CMF showed that in those patients who received tamoxifen the expression of *bcl-2* protein enhanced the predictive power of ER in identifying those patients who are likely to benefit from adjuvant antiestrogen therapy. In particular, among ER-negative tumors the expression of *bcl-2* seems to be able to identify a subgroup of patients, those *bcl-2* positive, who may receive some benefit from this form of therapy, whereas those with *bcl-2*-negative and ER-negative tumors have a very poor outcome.

Multivariate analysis performed in those patients who received adjuvant chemotherapy showed that *bcl-2*, tumor size, and nodes were significant and independent predictors for OS. For RFS tumor size, the number of metastatic nodes and PgR expression were significant and independent predictors. Thus, PgR seems to play a role in the chemotherapy-treated patients. This may be related to the fact that PgR-positive tumors are very well differentiated, have a slower growth rate, and therefore recurrences take longer to occur. Moreover, particularly in premenopausal women, adjuvant chemotherapy reduces ovarian function and those patients with PgR have functioning ERs. Thus, in these patients it may be a combined effect from chemotherapy of both hormone suppression and direct antitumor effect. Overall, patients with *bcl-2*-positive tumors were those who exhibited the

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greater benefit from both adjuvant treatments. This is in contrast to our expectation of poor response to adjuvant therapy with *bcl-2* expression.

This may be because the role of *bcl-2* appears to differ in different types of neoplasm. Initial studies suggested that in non-Hodgkin's lymphoma, patients with *bcl-2* rearrangements had a poorer response to therapy than those without such a rearrangement (48). However, a larger survey of follicular lymphomas did not show such a relationship (49) and in high-grade B cell lymphomas, *bcl-2* expression alone did not correlate with prognosis (50). Thus, one particular oncogene out of many genetic changes does not necessarily have to correlate with outcome. However, in normal B cell and T cell development, *bcl-2* expression is related to long-term memory cells which are able to undergo proliferation in response to suitable antigenic stimuli (51).

In contrast, *bcl-2* expression in normal glandular epithelium occurs in the cells that undergo hyperplasia and involution in response to hormonal stimuli (21, 52). In organized stratified epithelium, *bcl-2* is restricted to stem cells and proliferation zones, as it is in the lower crypts of the intestine and in the basal layer of the epidermis (21, 53). Thus, even in epithelia it has a different localization and is required for presumably different functions.

In non-small cell lung cancer, expression of *bcl-2* is associated with a better prognosis than *bcl-2* negativity although no patients in that study were treated with chemotherapy or radiotherapy and hence interactions with these modalities of treatment could not be assessed (25). It is possible that *bcl-2* suppresses apoptosis that would be induced by normal p53 (54), and is associated with slower growth than oncogenes that directly cause cell proliferation such as epidermal growth factor receptor or ras mutations. Thus, in tumors arising from epithelia with expression of *bcl-2* in basal layers, good prognosis may be due to a less aggressive mechanism of tranformation.

With regard to expression of *bcl-2* in endocrine-regulated tissues, hormone-independent prostate cancer has been associated with *bcl-2* expression (55). However, *bcl-2* is normally expressed in the basal stem cell layer which is not hormonally dependent (56). Thus, association with hormone resistance is understandable.

bcl-2 expression in breast cancer may be a reflection on the degree of differentiation of the tumor and although it was not associated particularly with well-differentiated tumors, *bcl-2* expression may be related to a phenotype more similar to normal tissue. In this case a better prognosis would be likely. Thus, *bcl-2*-positive breast cancer may simply be associated with a better differentiated tumor type. Tumors that are ER negative and *bcl-2* negative thus are poorly differentiated and will not respond to tamoxifen because of lack of ER. They could have the poorest prognosis. ER-positive cases, although *bcl-2* positive, may still have a *bcl-2* gene that can be regulated by estrogens and therefore responsive to tamoxifen.

Finally, it is known that there are multiple genetic pathways that control apoptosis, not all regulated by *bcl-2*. A family of genes have now been shown to be involved. *bcl-2* intereacts with bcl-x or bax to produce heterodimers (57–59). These can antagonize the effects of *bcl-2* on induction of apoptosis and have not, thus far, been studied in human cancers. Thus, association of *bcl-2* with apoptosis and prognosis may vary from one tumor type to another. A diverse co-expression of the genes involved in the control of apoptosis may explain the different action that *bcl-2* may have in different types of tumor (60).

Our study shows that *bcl-2* is a significant predictive factor for node-positive breast cancer. However, in our series it was not possible to distinguish whether *bcl-2*, as well as the other markers tested, were predictive of responsiveness to the treatments administered or if they were merely prognostic indicators. This is due to the fact that all of the patients studied received adjuvant therapy which influenced their outcome. The comparison between prognostic and predictive marker needs to be verified in a randomized clinical trial in which the same marker is determined in an arm of untreated patients (to see its prognostic value) *versus* a treated arm (to see its predictive value). This comparison seems to be feasible and advocable for node-negative breast cancer patients. However, it is difficult to find an untreated control group for patients with node-positive breast cancer because systemic adjuvant therapy is now the conventional therapy.

Our results are contrary to data from lymphoid cell lines and other epithelial cells, where drug resistance would be expected among *bcl-2*-positive tumors (24). This highlights the need to apply experimental *in vitro* molecular studies to the clinical situation.

bcl-2 would be useful in combination with node status to select patients for alternative treatments with existing therapeutic agents such as high-dose chemotherapy with stem cells support or more novel therapeutic strategies involving immunomodulation, differentiation, and anti-angiogenesis.

It is clear that the role of *bcl-2*, and of the other genes involved in the complex molecular control of apoptosis, may be different in diverse types of cancers, but that this can generate further hypotheses for experimental assessment of their normal role.

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