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Prognostic value of *bcl-2* expression in invasive breast cancer

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Summary Expression of the *bcl-2* proto-oncogene was studied immunohistochemically in 251 invasive ductal breast carcinomas (median follow-up time 91 months, range 24-186 months) and the results were correlated with clinicopathological data and prognostic variables. Sixty-three (25%) tumours were scored bcl-2 negative and 188 (75%) tumours were bcl-2 positive. No relationship could be observed between bcl-2 status and tumour grade, pTNM staging or menopausal status. A strong positive relationship was demonstrated between *bcl-2* immunoreactivity and oestrogen receptor status ($P<0.001$) and progesterone receptor status ($P<0.001$). No prognostic value was demonstrated for *bcl-2* expression on disease-free survival and overall survival in axillary node-negative breast cancer patients. However, in axillary node-positive breast cancer patients multivariate analysis demonstrated absence of *bcl-2* expression to be independently related to shortened disease-free survival ($P=0.003$) and shortened overall survival ($P<0.001$). Our results suggest a potential important role for *bcl-2* expression as a modulator of response to adjuvant therapy in breast cancer.

Keywords: *bcl-2*; immunohistochemistry; prognosis; breast cancer

Cloning of the t(14;18) chromosomal breakpoint in follicular lymphoma led to the discovery of the *bcl-2* proto-oncogene (Tsujimoto *et al.*, 1984; Cleary *et al.*, 1986; Tsujimoto and Croce, 1986). Gene transfer experiments have demonstrated a role for *bcl-2* in preventing apoptosis in growth factor-deprived haemopoietic cell lines (Vaux *et al.*, 1988; Nuñez *et al.*, 1990) and in neurotrophic factor-deprived neurons (Garcia *et al.*, 1992; Allsopp *et al.*, 1993). In transgenic mice *bcl-2* has been shown to prolong cell survival (McDonnell *et al.*, 1989; Strasser *et al.*, 1990). Antisense-mediated inhibition of *bcl-2* gene expression reduces leukaemic cell growth and survival in an *in vitro* setting (Reed *et al.*, 1990). Hockenberry *et al.* (1990) concluded *bcl-2* to be unique among proto-oncogenes by its ability to block programmed cell death without promoting cell proliferation, which led to its categorisation as a member of a new category of oncogenes: regulators of cell death (Korsmeyer, 1992).

The 25 kDA bcl-2 protein contains a hydrophobic COOH-terminal region allowing post-translational insertion into intracellular membranes and orientation towards the cytosol (Chen-Levy and Cleary, 1990). Studies on the subcellular location of the bcl-2 protein have demonstrated its residence in the nuclear envelope, parts of the endoplasmic reticulum, the outer mitochondrial membrane and to a lesser extent in the plasma membrane (Krajewski *et al.*, 1993; Akao *et al.*, 1994; de Jong *et al.*, 1994). Although its exact biochemical mechanism of action remains largely unexplained, an important physiological role for bcl-2 in cell development and differentiation, in tissue homeostasis and in morphogenesis was shown in immunohistochemical studies on fetal and adult human tissues of different origin (Hockenberry *et al.*, 1991; LeBrun *et al.*, 1993; Lu *et al.*, 1993).

Immunohistochemical studies on *bcl-2* expression in human breast cancer have demonstrated a strong association with oestrogen receptor (ER) status, illustrating the possibility that *bcl-2* is an ER regulated gene (Bhargava *et al.*, 1994; Chan *et al.*, 1993; Doglioni *et al.*, 1994; Gee *et al.*, 1994; Leek *et al.*, 1994; Nathan *et al.*, 1994; Silvestrini *et al.*, 1994). The presence of bcl-2 protein immunostaining has been shown to be associated with a low apoptotic index in malignant mammary epithelium (Chan *et al.*, 1993). Leek *et al.* (1994) did not demonstrate a correlation between *bcl-2* status and tumour size, nodal status, tumour grade and histological type in 111 breast carcinomas. By contrast other authors correlated *bcl-2* immunoreactivity with larger tumour size, with the lobular type and with better differentiated neoplasms (Bhargava *et al.*, 1994; Doglioni *et al.*, 1994; Joensuu *et al.*, 1994; Silvestrini *et al.*, 1994). An inverse correlation was noticed between bcl-2 protein expression and proliferative activity as measured by Ki 67 immunostaining and [^{3}H]thymidine labelling index (Doglioni *et al.*, 1994; Silvestrini *et al.*, 1994). Furthermore, loss of *bcl-2* expression has been associated with presence of a range of molecular markers of poor prognosis in breast cancer, including epidermal growth factor receptor (EGFR), c-erb-B2 and p53 (Doglioni *et al.*, 1994; Gee *et al.*, 1994; Joensuu *et al.*, 1994; Leek *et al.*, 1994; Nathan *et al.*, 1994; Silvestrini *et al.*, 1994). In a recently published study on *bcl-2* expression in axillary node-negative breast cancer, no prognostic role for *bcl-2* on 6 year relapse-free and overall survival was retained following multivariate analysis (Silvestrini *et al.*, 1994). In a series of 174 breast cancers with long-term follow-up, which were primarily treated by surgery with or without locoregional radiotherapy, no prognostic role for *bcl-2* expression was observed following multivariate analysis (Joensuu *et al.*, 1994).

The aim of the present study was to determine immunohistochemically the expression of the *bcl-2* proto-oncogene in a series of invasive ductal breast cancers and to evaluate the prognostic value of *bcl-2* expression in axillary node-negative and axillary node-positive breast cancer.

Materials and methods

Patients and follow-up

The study group consisted of 251 women who underwent surgery for primary invasive ductal breast carcinoma between March 1979 and June 1992 at the Antwerp University Hospital. The median age of the patients at the time of diagnosis was 56 years (range 27-89 years). All patients had a preoperative chest radiograph, bone scintigraphy, ultrasound scan of the liver and blood test (full blood count, liver function tests, carcinoembryonic antigen). If there was no evidence of metastatic disease they were surgically treated by modified radical mastectomy or wide local excision of the primary tumour with axillary lymphadenectomy. All patients who had breast conserving surgery received adjuvant radiotherapy. Patients were pathologically staged according

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to the UICC (1992) *TNM Atlas* criteria. All tumours were histologically classified as invasive ductal breast carcinomas and graded according to the methodology of Bloom and Richardson (1957). Data on tumour grade, tumour size, nodal status, presence or absence of metastatic disease and menopausal status are given in Table I. Menopausal status was assessed using serum gonadotrophin and oestradiol measurements in perimenopausal patients. Axillary node-negative patients were followed conservatively and received no adjuvant treatment. Axillary node-positive premenopausal patients had six cycles of CMF (cyclophosphamide, methotrexate and 5-fluorouracil) polychemotherapy. Axillary node-positive post-menopausal patients received adjuvant endocrine treatment (tamoxifen, 20 mg day^{-1} orally). All patients underwent a follow-up physical examination every 6 months and had further investigations if they developed symptoms or signs suggestive of recurrent or metastatic disease. The median follow-up time in our study group was 91 months (range 24–186 months). Ethical committee approval was sought and received for this clinical study.

Immunohistochemistry

Formalin-fixed, paraffin-embedded representative primary tumour samples were available for all patients. Five-micron-thick sections were cut and mounted onto 3-aminopropyltriethoxysilane-coated glass slides. They were dewaxed in xylene followed by rehydration in decreasing ethanol series, water and phosphate-buffered saline (PBS) pH 7.4. Endogenous peroxidase activity was quenched in 0.3% hydrogen peroxide in 100% methanol, followed by rehydration through graded ethanol and distilled water. Subsequently an antigen retrieval procedure for formalin-fixed paraffin sections was performed by immersion of the slides in 10 mM citrate buffer (10 mM citrate monohydrate in distilled water, pH 6.0) and exposure to microwave irradiation twice for 5 min with a cooling period of 3 min in between the sessions (Cattoretti *et al.*, 1992). After cooling to room temperature slides were removed to PBS and preincubated with 10% normal rabbit serum (Dako, Denmark) to reduce non-specific binding. The sections were incubated overnight at 4°C with monoclonal mouse anti-human bcl-2 oncoprotein (clone 124, isotype IgG1; Dako) diluted 1:40 in PBS supplemented with 1% bovine serum albumin. This monoclonal mouse antibody is directed towards a synthetic peptide comprising amino acids 41–54 of the human bcl-2 protein (Cleary *et al.*, 1986; Tusjimoto and Croce, 1986; Pezzella *et al.*, 1990). Its efficacy has been proven on frozen sections and on paraffin sections (Pezzella *et al.*, 1990, 1992, 1993; LeBrun *et al.*, 1992; Leek *et al.*, 1994; Silvestrini *et al.*, 1994). The sections were then overlaid with biotinylated rabbit anti-mouse polyclonal antibody (Dako) diluted 1:300. Binding was detected by applying the avidin–biotin–peroxidase complex (Dako). DAB (3-3'-diaminobenzidine tetrahydrochloride) was used as chromogen and Mayer's haematoxylin was used as counterstain. Negative controls were performed by omitting the primary antibody and by substituting the anti-bcl-2 antibody for an unrelated monoclonal antibody of the same isotype IgG1 in the same concentration but directed against an unrelated antigen (monoclonal mouse anti-human CD68 antibody, isotype IgG1; Dako). Sections of a follicular lymphoma were used as positive controls (Cleary *et al.*, 1986; Tusjimoto and Croce, 1986).

Bcl-2 cytoplasmic immunoreactivity was quantified by counting at least 1000 tumour cells in different random fields, using a high-power (400 ×) objective. Results were expressed as percentage of tumour cells staining positively for bcl-2. For further statistical analysis two groups of tumours were defined: tumours containaing 10% or less (bcl-2 negative) and tumours containing more than 10% positively staining tumour cells (bcl-2 positive). This cut-off value was chosen taking into account statistical guidelines for prognostic factor studies in oncology (Simon and Altman, 1994).

Table I Bcl-2 cytoplasmic immunoreactivity in relation to tumour grade, pTNM staging, ER status, PgR status and menopausal status

	Bcl-2 *cytoplasmic immunoreactivity* Bcl-2 *negative*	Bcl-2 *positive*	*Significance* P-*value*[a]
All tumours	63 (25%)	188 (75%)	
Tumour grade:			
Grade I	15 (24%)	56 (30%)	
Grade II	32 (51%)	85 (45%)	NS
Grade III	16 (25%)	47 (25%)	
Tumour size[b]			
pT1	28 (44%)	91 (48%)	
pT2	24 (38%)	67 (36%)	NS
pT3	6 (10%)	10 (5%)	
pT4	5 (8%)	20 (11%)	
Nodal status[b]			
pN0	33 (52%)	91 (48%)	
pN1	20 (32%)	64 (34%)	NS
pN2	9 (14%)	14 (8%)	
pNX	1 (2%)	19 (10%)	
Metastases[b]			
M0	62 (98%)	169 (90%)	NS*
M1	1 (2%)	19 (10%)	
ER status[c]			
Positive	22 (35%)	135 (72%)	<0.001*
Negative	41 (65%)	53 (28%)	
PgR status[d]			
Positive	26 (41%)	125 (67%)	<0.001*
Negative	37 (59%)	63 (33%)	
Menopause			
Pre	22 (35%)	63 (33%)	NS*
Post	41 (65%)	125 (67%)	

[a]Chi-squared test. [b]Pathological tumour staging according to UICC criteria. [c]Oestrogen receptor status. [d]Progesterone receptor status. *Yates' correction for small numbers. NS, not significant.

Quantification of steroid hormone receptors

Oestrogen receptor (ER) and progesterone receptor (PgR) were determined using an enzymatic assay (Abbott Enzyme Immunoassay-Oestrogen Receptor, Abbott Enzyme Immunoassay-Progesterone Receptor). Results were expressed quantitatively as amount of receptor protein per gram of tissue (fmol g^{-1}). Values greater than 20 fmol g^{-1} tissue protein were considered positive.

Statistical analysis

A chi-squared test was performed to evaluate the relationship between bcl-2 immunoreactivity and tumour grade, tumour size, nodal status, presence or absence of metastases, oestrogen receptor (ER) status, progesterone receptor (PR) status and menopausal status.

Overall survival curves and disease-free survival curves, starting from the date of surgery, were plotted using the Kaplan and Meier (1958) method and their statistical significance was calculated by use of the log-rank test. Locoregional disease relapse and/or distant metastases were considered end points for disease-free survival. Cox's (1972) proportional hazard regression analysis was used for multivariate analysis and for calculation of the hazard ratios and their confidence intervals.

For all statistical analyses a P-value <0.05 was considered statistically significant.

Results

Tissue distribution of bcl-2 immunoreactivity

Sixty-three (25%) tumours were scored as bcl-2 negative and 188 (75%) tumours were scored as bcl-2 positive. In bcl-2-positive cases no relationship was observed between location of bcl-2 immunoreactivity and definite neoplastic areas

(tumour centre *vs* infiltrative margins). In all tumour sections stromal fibroblasts were bcl-2 negative and lymphocytes were bcl-2 positive. Cytoplasmic immunoreactivity for bcl-2 protein was always observed in normal mammary glandular tissue in those sections containing normal breast tissue adjacent to the tumour.

Association with clinicopathological variables

Correlations between bcl-2 immunoreactivity and clinicopathological variables are shown in Table I. No relationship was demonstrated between bcl-2 immunoreactivity and tumour grade, tumour size, nodal status, presence or absence of metastases or menopausal status. A significant positive relationship was found between bcl-2 immunoreactivity and oestrogen receptor status ($P<0.001$) or progesterone receptor status ($P<0.001$).

Prognostic relevance

In the group of patients initially staged as M0 ($n = 231$) (median follow-up time 91 months, range 24–186 months) univariate analysis demonstrated a significantly shorter disease-free survival (log-rank test, $P<0.001$) and a significantly shorter overall survival (log-rank test, $P<0.001$) in bcl-2-negative tumours *vs* bcl-2-positive tumours (Figure 1). The joint effect of bcl-2 status, tumour grade, tumour size, nodal status and ER status on disease-free survival and overall survival as evaluated by Cox regression analysis is given in Table II. In multivariate analysis bcl-2 status, tumour grade, tumour size and nodal status were indicators for disease-free survival and, with the exception of tumour size, overall survival. Cox regression analysis following adjustment for tumour grade, tumour size, nodal status and ER status demonstrated a significantly shorter disease-free survival (adjusted hazard ratio = 2.08, 95% CI 1.25–3.45, $P = 0.005$) and a significantly shorter overall survival (adjusted hazard ratio = 2.49, 95% CI 1.43–4.33, $P = 0.001$) in *bcl*-2-negative tumours *vs* *bcl*-2-positive tumours.

In the group of axillary node-negative patients ($n = 124$) (median follow-up time 84 months, range 24–161 months) univariate analysis revealed no statistical difference in disease-free survival (log-rank test, $P = 0.077$) or in overall survival (log-rank test, $P = 0.139$) between bcl-2-negative and bcl-2-positive tumours (Figure 2). The joint effect of *bcl*-2 status, tumour grade, tumour size and ER status on disease-free survival and overall survival as evaluated by Cox regression analysis is given in Table III. Multivariate analysis by Cox regression analysis following adjustment for tumour grade, tumour size and ER status did not show a statistical difference in disease-free survival (adjusted hazard ratio = 1.52, 95% CI 0.64–3.58, $P = 0.351$) or in overall survival (adjusted hazard ratio = 1.63, 95% CI 0.63–4.22, $P = 0.326$) between bcl-2-negative tumours and bcl-2-positive tumours.

In the group of axillary node-positive patients ($n = 107$)

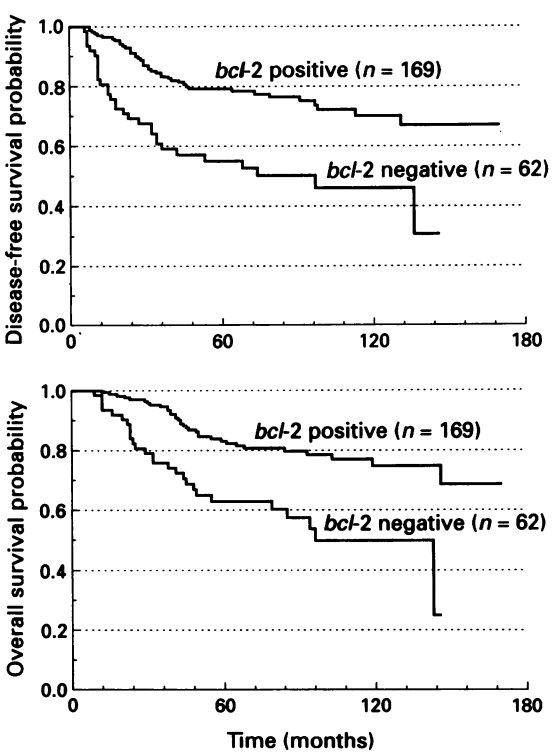

Figure 1 Kaplan–Meier life table analysis for disease-free survival (log-rank test, $P<0.001$) and for overall survival (log-rank test, $P<0.001$) in patients initially staged as M0 ($n = 231$).

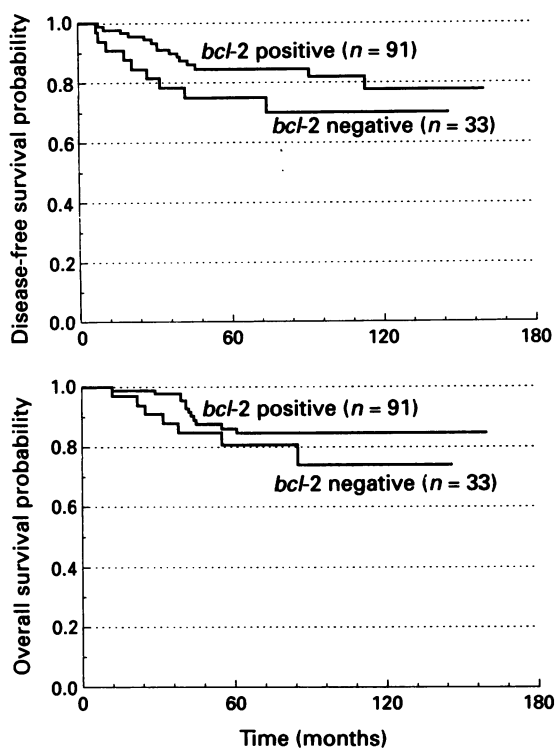

Figure 2 Kaplan–Meier life table analysis for disease-free survival (log-rank test, $P = 0.077$) and for overall survival (log-rank test, $P = 0.139$) in axillary node-negative patients ($n = 124$).

Table II Multivariate analysis of disease-free survival and overall survival in 231 patients initially staged as M0 (Cox regression analysis)

	Disease-free survival			*Overall survival*		
	HR[a] (95% CI)	*LRS[b]*	P	HR[a] *(95% CI)*	*LRS[b]*	P
Bcl-2 expression (negative[c] *vs* positive)	2.08 (1.25–3.45)	7.78	0.005	2.49 (1.43–4.33)	10.16	0.001
Tumour grade (I, II, II)[d]	1.45 (1.04–2.01)	4.92	0.026	2.15 (1.48–3.12)	17.58	<0.001
Tumour size (pT1, pT2, pT3, pT4)[d]	1.32 (1.01–1.73)	4.02	0.045	1.21 (0.90–1.62)	1.61	0.205
Nodal status (pN0, pN1, pN2)[d]	1.69 (1.20–2.37)	8.97	0.003	1.66 (1.13–2.44)	6.71	0.01
ER status (positive[c] *vs* negative)	0.66 (0.40–1.10)	2.55	0.111	0.78 (0.45–1.35)	0.79	0.374

[a]Adjusted hazards ratio of relapsing or dying (95% confidence intervals). [b]Likelihood ratio statistic on one degree of freedom. [c]Reference category. [d]Mean hazard ratio between two adjacent categories.

(median follow-up time: 101 months, range 32–186 months) univariate analysis demonstrated a significantly shorter disease-free survival (log-rank test, $P<0.001$) and a significantly shorter overall survival (log-rank test, $P<0.001$) in bcl-2-negative tumours than in bcl-2-positive tumours (Figure 3). The joint effect of bcl-2 status, tumour grade, tumour size and ER status on disease-free survival and overall survival as evaluated by Cox regression analysis is given in Table IV. In multivariate analysis bcl-2 status, tumour grade and tumour size were independent indicators of disease-free survival and, with the exception of tumour size, overall survival. Multivariate analysis by Cox regression analysis following adjustment for tumour grade, tumour size and ER status demonstrated a significantly shorter disease-free survival (adjusted hazard ratio = 2.82, 95% CI 1.41–5.64, $P = 0.003$) and a significantly shorter overall survival (adjusted hazard ratio = 3.76, 95% CI 1.78–7.92, $P<0.001$) in bcl-2-negative tumours than in bcl-2-positive tumours.

It needs to be emphasised that our survival curves for disease-free survival and overall survival in the different patient groups have been plotted up to 180 months, although the median follow-up period in our study group was 91 months. Owing to the limited number of patients at risk at the end of the curves, disease-free survival rates and overall survival rates beyond 130 months follow-up time should be interpreted with caution (Figures 1–3).

Discussion

The *bcl-2* proto-oncogene has been demonstrated to be an inhibitor of programmed cell death without promoting cell proliferation (Hockenberry *et al.*, 1990). We studied the expression of the *bcl-2* gene in a series of 251 invasive ductal breast carcinomas and correlated its expression with clinicopathological data and prognosis. Cytoplasmic immunoreactivity for *bcl-2* in more than 10% of tumour cells was present in 188 (75%) tumours, which were considered *bcl-2* positive. Sixty-three (25%) tumours containing 10% or fewer positively staining tumour cells were considered *bcl-2* negative. We could not demonstrate a relationship between *bcl-2* immunoreactivity and tumour grade, tumour size, nodal status, presence or absence of metastases and menopausal status.

In the present study we observed a strong positive relationship between *bcl-2* immunoreactivity and oestrogen and progesterone receptor status. This is in agreement with previously published data (Chan *et al.*, 1993; Bhargava *et al.*, 1994; Doglioni *et al.*, 1994; Gee *et al.*, 1994; Leek *et al.*, 1994; Nathan *et al.*, 1994; Silvestrini *et al.*, 1994). These observations support the hypothesis that *bcl-2* expression in breast carcinoma may be an oestrogen receptor-regulated phenomenon.

Silvestrini *et al.* (1994) studied the prognostic value of the bcl-2 oncoprotein on 6 year relapse-free and overall survival in 283 axillary node-negative breast cancer patients. Univariate analysis demonstrated low bcl-2 immunoreactivity to be associated with shortened relapse-free and overall survival. Multivariate analysis including bcl-2 status, tumour size [^{3}H]thymidine labelling index and ER status demonstrated an independent prognostic role for *bcl-2* expression. However, no prognostic role for *bcl-2* expression on 6 year relapse-free survival and overall survival was retained when p53 expression was included in the multivariate analysis (Silvestrini *et al.*, 1994). In a series of 174 women with breast cancer treated with radical surgery with or without locoregional radiotherapy and with very long-term follow-up (median 31 years), a significant association was demonstrated

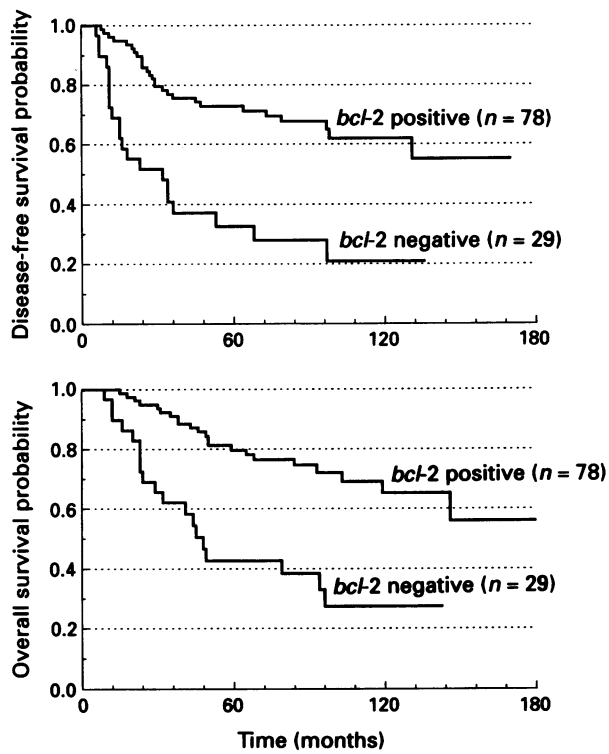

Figure 3 Kaplan–Meier life table analysis for disease-free survival (log-rank test, $P<0.001$) and for overall survival (log-rank test, $P<0.001$) in axillary node-positive patients ($n = 107$).

Table III Multivariate analysis of disease-free survival and overall survival in 124 axillary node-negative patients (Cox regression analysis)

	Disease-free survival			*Overall survival*		
	HR[a] *(95% CI)*	*LRS*[b]	P	*HR*[a] *(95% CI)*	*LRS*[b]	P
Bcl-2 expression (negative[c] *vs* positive)	1.52 (0.64–3.58)	0.87	0.351	1.63 (0.63–4.22)	0.96	0.326
Tumour grade (I, II, II)[d]	1.06 (0.62–1.82)	0.05	0.818	1.84 (0.97–3.53)	3.55	0.059
Tumour size (pT1, pT2, pT3, pT4)[d]	1.33 (0.80–2.22)	1.09	0.296	1.47 (0.85–2.53)	1.69	0.194
ER status (positive[c] *vs* negative)	0.37 (0.16–0.88)	5.16	0.023	0.53 (0.21–1.35)	1.80	0.180

[a]Adjusted hazards ratio of relapsing or dying (95% confidence intervals). [b]Likelihood ratio statistic on one degree of freedom. [c]Reference category. [d]Mean hazard ratio between two adjacent categories.

Table IV Multivariate analysis of disease-free survival and overall survival in 107 axillary node-positive patients (Cox regression analysis)

	Disease-free survival			*Overall survival*		
	HR[a] *(95% CI)*	*LRS*[b]	P	*HR*[a] *(95% CI)*	*LRS*[b]	P
Bcl-2 expression (negative[c] *vs* positive)	2.82 (1.41–5.64)	8.59	0.003	3.76 (1.78–7.92)	12.34	<0.001
Tumour grade (I, II, II)[d]	1.88 (1.22–2.93)	8.40	0.004	2.53 (1.55–4.12)	15.18	<0.001
Tumour size (pT1, pT2, pT3, pT4)[d]	1.42 (1.05–1.92)	4.97	0.026	1.19 (0.87–1.63)	1.21	0.271
ER status (positive[c] *vs* negative)	1.02 (0.51–2.04)	0.002	0.962	0.98 (0.46–2.08)	0.003	0.960

[a]Adjusted hazards ratio of relapsing or dying (95% confidence intervals). [b]Likelihood ratio statistic on one degree of freedom. [c]Reference category. [d]Mean hazard ratio between two adjacent categories.

between low *bcl*-2 expression and shortened overall survival following univariate analysis in axillary node-positive but not in axillary node-negative patients. Following multivariate analysis no independent prognostic role for *bcl*-2 expression was retained in axillary node-negative or in axillary node-positive patients (Joensuu *et al.*, 1994). In the present series we studied the prognostic value of *bcl*-2 expression on disease-free survival and overall survival in 231 breast cancer patients initially staged as M0. Univariate analysis and multivariate analysis including established prognostic factors in breast carcinoma demonstrated absence of *bcl*-2 expression to be independently associated with shortened disease-free survival and shortened overall survival in axillary node-positive breast cancer but not in axillary node-negative breast cancer. The observed prognostic role for *bcl*-2 expression in the complete study population mainly reflects its strong prognostic value in axillary node-positive breast cancer. Our study results in part confirm the results obtained in previous studies but demonstrate an intriguing prognostic role for *bcl*-2 expression in axillary node-positive breast cancer.

In follicular lymphoma the presence of *bcl*-2 overexpression as a consequence of the t(14;18) translocation has no prognostic value and appears to be a hallmark for slow tumour progression (Pezzella *et al.*, 1992). In non-small-cell lung carcinoma the observation of a group of *bcl*-2-positive tumours with relatively slow disease progression suggests a role for *bcl*-2 expression as an initial oncogenic effect leading to less aggressive tumour growth as observed in follicular lymphoma (Pezzella *et al.*, 1993). The observed difference in independent prognostic power for *bcl*-2 expression between axillary node-negative and axillary node-positive patients in our series is extremely interesting and needs further elucidation. *In vitro* and *in vivo* studies have demonstrated a role for the bcl-2 protein in the prevention of apoptosis induced by anti-cancer drugs (Campos *et al.*, 1993; Miyashita and Reed, 1993). *Bcl*-2 transfection has been demonstrated to confer resistance to anti-cancer agents by non-conventional drug-resistance pathways (Fisher *et al.*, 1993). A recent study in breast cancer has reported bcl-2 immunostaining to be a better predictor for response to systemic endocrine therapy than oestrogen receptor status in a limited series of patients who only received endocrine therapy following primary surgery (Gee *et al.*, 1994). In contrast to our findings, Joensuu *et al.* (1994) could not demonstrate a prognostic role for *bcl*-2 expression on overall survival in axillary node-positive breast cancer patients primarily treated by radical surgery with or without locoregional radiotherapy and in whom no adjuvant therapy was administered. These data and our study results strongly suggest a role for *bcl*-2 expression as a predictor for response to chemotherapy or endocrine therapy in breast cancer patients. However, investigating such interactions in our study population is problematic because of the need for subgroup analysis in limited numbers of patients and because of the heterogeneous character of second-line and third-line treatment in those patients developing locoregional disease relapse and/or distant metastases which consists of various combinations of chemotherapy and/or endocrine therapy and/or radiotherapy. Larger patient groups with better standardised curative and palliative treatment protocols are needed to study the role of *bcl*-2 as a modulator of response to chemotherapy or endocrine therapy in breast cancer.

To what extent *bcl*-2 expression influences apoptotic cell death rate in solid neoplasms and its final effect on disease progression remains to be resolved. Recently, cDNAs have been cloned for several novel human genes, revealing a family of bcl-2 related proteins. The bax protein has been demonstrated to promote apoptosis by opposing bcl-2's function through heterodimerisation (Oltvai *et al.*, 1993). Bcl-x_L has been shown to inhibit apoptosis, whereas its shorter alternative splice form bcl-x_S, promotes apoptosis (Boise *et al.*, 1993). The *MCL*1 gene and the *A*1 gene have been demonstrated to have sequence similarity with the *bcl*-2 gene, although their function remains unknown (Kozopas *et al.*, 1993; Lin *et al.*, 1993). Other non-bcl-2-related proteins playing a role in the regulation of programmed cell death have been isolated (Gagliardini *et al.*, 1993; Nakashima *et al.*, 1993).

Other important oncogenes and tumour-suppressor genes play a role in the regulation of apoptosis. c-*myc*, although generally considered as an important element in proliferation control, induces apoptosis in specific conditions (Evan *et al.*, 1992; Shi *et al.*, 1992). The action of c-*myc* as a stimulator of both mitogenesis and apoptosis appears to be dependent on the presence of *bcl*-2 (Bissonnette *et al.*, 1992; Fanidi *et al.*, 1992). Wild-type p53 has been demonstrated to be able to induce apoptosis (Yonis-Rouach *et al.*, 1991). The *bcl*-2 gene is a transcriptional target for wild-type p53. Wild-type p53 decreases bcl-2 protein levels and increases bax protein levels *in vitro* and *in vivo*, explaining the ability of p53 to induce apoptotic cell death (Miyashita *et al.*, 1994*a*,*b*). Furthermore, in breast cancer cell lines mutant p53 induces down-regulation of *bcl*-2 at both mRNA and protein level (Haldar *et al.*, 1994). The critical role played by p53 in the carcinogenesis of human breast cancer is well documented. About 30–50% of human breast cancers carry a mutant p53 gene and about an additional 30% carry non-functional wild-type p53 sequestered in the cytoplasm of tumour cells (Moll *et al.*, 1992). The presence of mutated or overexpressed p53 has been associated with poor prognostic markers, such as high histopathological grading, high levels of Ki67 or EGFR and the absence of hormone receptors, as well as with shortened disease-free survival and/or overall survival (Cattoretti *et al.*, 1988; Isola *et al.*, 1992; Poller *et al.*, 1992; Thor *et al.*, 1992; Allred *et al.*, 1993). The inverse correlation between *bcl*-2 expression and the presence of immunohistochemically demonstrable mutant p53 observed in breast cancer could reflect down-regulation of *bcl*-2 by mutant p53 (Doglioni *et al.*, 1994; Joensuu *et al.*, 1994; Leek *et al.*, 1994; Silvestrini *et al.*, 1994).

We conclude absence of *bcl*-2 expression to be an independent marker of poor prognosis in axillary node-positive breast cancer. Absence of *bcl*-2 expression in invasive ductal breast carcinoma possibly reflects down-regulation of the *bcl*-2 gene by mutant p53. Our results suggest a potentially important role for *bcl*-2 as a modulator of response to adjuvant therapy in breast cancer.

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