Bcl-2 Is a Prognostic Marker in Breast Cancer Independently of the Nottingham Prognostic Index

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Abstract

Purpose: Prognostication of breast cancer using clinicopathologic variables, although useful, remains imperfect. Many reports suggest that gene expression profiling can refine the current approach. Alternatively, it has been shown that panels of proteins assessed by immunohistochemistry might also be useful in this regard. We evaluate the prognostic potential of a panel of markers by immunohistochemistry in a large case series to establish if either a single marker or a panel could improve the prognostic power of the Nottingham Prognostic Index (NPI). We validated the results in an independent series.

Experimental Design and Results: The expression of 13 biomarkers was evaluated in 930 breast cancers on a tissue microarray. Eight markers [estrogen receptor (ER), progesterone receptor (PR), Bcl-2, cyclin E, p53, MIB-1, cytokeratin 5/6, and HER2] showed a significant association with survival at 10 years on univariate analysis. On multivariate analysis that included these eight markers and the NPI, only the NPI [hazard ratio (HR), 1.35; 95% confidence interval (95% CI), 1.16-1.56; $P = 0.0005$], ER (HR, 0.59; 95% CI, 0.39-0.88; $P = 0.011$), and Bcl-2 (HR, 0.68; 95% CI, 0.46-0.99; $P = 0.055$) were significant. In a subsequent multivariate analysis that included the NPI, ER, and Bcl-2, only Bcl-2 (HR, 0.62; 95% CI, 0.44-0.87; $P = 0.006$) remained independent of NPI (HR, 1.50; 95% CI, 1.16-1.56; $P = 0.004$). In addition, Bcl-2, used as a single marker, was more powerful than the use of a panel of markers. Based on these results, an independent series was used to validate the prognostic significance of Bcl-2. ER and PR were also evaluated in this validation series. Bcl-2 (HR, 0.83; 95% CI, 0.71-0.96; $P = 0.018$) retained prognostic significance independent of the NPI (HR, 2.04; 95% CI, 1.67-2.51; $P < 0.001$) with an effect that was maximal in the first 5 years.

Conclusion: Bcl-2 is an independent predictor of breast cancer outcome and seems to be useful as a prognostic adjunct to the NPI, particularly in the first 5 years after diagnosis.

One of the greatest challenges in breast cancer management is to accurately predict outcome for each patient so that we can determine who will benefit from adjuvant therapy. To do this at present, we rely heavily on traditional pathologic variables, such as lymph node status (1, 2), tumor size (1), and tumor grade (3, 4). In many centers, these variables are combined into the Nottingham Prognostic Index (NPI) to generate a prognostic score for each patient that is more predictive of outcome than any one individual feature (5). However, despite the broad applicability of clinicopathologic indices, such as the NPI, they cannot accurately predict outcome for all patients (6–8) and we are still unable, for example, to separate the 30% of node-negative patients who will relapse from the 70% who will not; as a result, many patients receive unnecessary adjuvant treatment.

It has been suggested that gene expression microarray studies offer the greatest promise for refining prognostication in breast cancer. In the last 6 years, a molecular taxonomy of breast cancer has been produced (9) and reports have suggested that gene expression profiles have more prognostic power than traditional prognostic methods (10–13). Optimism must be guarded, however, as expression microarray studies are labor

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Table 1. Characteristics of UBC series (N = 930)

Variable	n*	Cases (%)
Tumor type	855	
Ductal		755 (88)
Lobular		47 (6)
Other		53 (6)
Grade	727	
1		68 (9)
2		234 (32)
3		425 (59)
ER status	859	
Positive		395 (46)
Negative		464 (54)
Tumor size (mm)	921	
≤20		347 (38)
>20		420 (45)
>50		154 (18)
Nodal status	800	
Negative		238 (30)
Positive (1-3 nodes)		332 (41)
Positive (>3 nodes)		230 (29)
NPI	557	
GPG		34 (6)
MPG		236 (42)
PPG		287 (52)
Survival at 10 y	930	
Alive and well		257 (28)
Alive with breast disease		36 (4)
Alive with other malignancy		9 (1)
Dead from disease		476 (51)
Dead from other cause		59 (6)
Dead from second malignancy		93 (10)

*Number of cases for which data were available.

intensive and their applicability outside the research setting is uncertain. Furthermore, the genes that have been included in the prognostic classifiers generated from array-based studies have varied tremendously and results still need to be validated in large-scale studies (14, 15).

We (16) and others (17–21) have used protein expression profiling by immunohistochemistry on tissue microarrays as a practical alternative for refining classification and prognostication in invasive breast cancer. In this article, we present data from a developmental study to test this methodology and a validation study using an independent series. We described previously a subclassification of breast cancer that was similar to that produced by expression microarray studies (12) based on data from only 13 protein markers analyzed by immunohistochemistry (16). In the work reported here, the performance of this 13–protein biomarker classifier for predicting long-term survival in breast cancer was evaluated. Analysis of the 13 protein markers in >930 cases (developmental study) revealed that an unsupervised clustering-based classification using a panel of markers did no better than the NPI in predicting long-term outcome. However, one marker (Bcl-2) used alone improved the prognostic power of the NPI. We then tested the independent prognostic significance of Bcl-2 in an independent large series (validation study).

Materials and Methods

Case selection. The University of British Columbia (UBC) case series used for the developmental study was from a cohort of 2,154 women with stage I to III breast cancer who participated in four different British Columbia Cancer Agency clinical trials between 1970 and 1990, and all received chemotherapy (22, 23). Nine hundred thirty cases were used to construct tissue microarrays based solely on the availability of paraffin-embedded tumor blocks, and in these, we evaluated the expression of 13 protein markers. The available clinical information included date of diagnosis, age at diagnosis (mean, 48.5 years; range, 22-90), date and type of relapse, and date and cause of death. For most patients, tumor size, histologic grade, tumor type, and nodal status were also available (Table 1). The study was approved by the Clinical Research Ethics Board of the UBC. All patients were followed up after the end of the original trial until 2001 (mean follow-up, 9.7 years; median, 8.7; range, 0.4-39.4).

The Nottingham case series used for the validation study consisted of 1,961 consecutive cases of primary operable breast carcinoma patients presenting from 1986 to 1998 and entered into the Nottingham Tenovus Primary Breast Carcinoma Series (Table 2). The majority of patients with estrogen receptor (ER)–positive disease received adjuvant hormone therapy and only a small number received adjuvant

Table 2. Characteristics of the Nottingham series

Variable	n*	Cases (%)
Tumor type	1,961	
NST		1,089 (56)
Lobular		219 (11)
Mixed NST and special type		443 (22)
Other		210 (11)
Tumor grade	1,940	
1		367 (19)
2		648 (33)
3		925 (48)
ER status	1, 812	
Positive		1,268 (70)
Negative		544 (30)
Tumor size (mm)	1,943	
<20		1,033 (53)
20-50		864 (45)
>50		46 (2)
Nodal status	1,938	
Negative		1,233 (64)
Positive (1-3 nodes)		549 (28)
Positive (>3 nodes)		156 (8)
NPI	1,934	
GPG		618 (32)
MPG		994 (51)
PPG		322 (17)
Survival at 10 y	1,933	
Alive and well		1,718 (89)
Dead from disease		187 (10)
Dead from other causes		27 (1)

*Number of cases for which data were available.

Table 3. Primary antibodies

Antibody	Clone	Source	Dilution	Antigen retrieval*
Bcl-2	124	Dako	1:20	Pressure cooker
HER2	CBE-356	Novocastra	1:40	Microwave†
c-Myc	9E11	Novocastra	1:150	Not used
Cyclin E	13A3	Novocastra	1:40	Pressure cooker
CK 5/6	D5/16B4	Dako	1:100	Pressure cooker
CK 17	E3	Novocastra	1:30	Pressure cooker
CK 8/18	5D3	Novocastra	1;100	Pressure cooker
ER	6F11/2	Novocastra	1:30	Pressure cooker
Ki-67	MIB-1	Dako	1:30	Pressure cooker
Mcm-2	BM28	Transduction Laboratories	1:1,000	Pressure cooker
p27	1B4	Novocastra	1:20	Pressure cooker
p53	DO-7	Dako	1:100	Pressure cooker
PR	PgR 636	Dako	1:50	Pressure cooker

*Microwave for 15 minutes in 850 W microwave; pressure cooker in 0.01 mol/L sodium citrate (pH 6) for 2 minutes.
†Low pH retrieval buffer (Dako).

chemotherapy. The mean survival was 62 months (median, 58; range, 1-192) with 10,077 years of person follow-up for 1,933 patients. Patient, clinical, and histologic data known included age at diagnosis (mean, 54 years; range, 18-70), menopausal status, tumor size, histologic grade (3), lymph node status, presence or absence of lymphovascular invasion, ER status, local and regional recurrence data as well as information on the development of metastatic disease, and date and cause of mortality.

Tissue microarray construction and immunohistochemistry. Tissue microarrays were constructed from both the UBC and the Nottingham series using a tissue microarrayer (Beecher Instruments, Sun Prairie, WI) as described previously (24). A single representative 0.6-mm tissue core was taken from each tumor block. Sections from the tissue microarrays were cut at 3.5 to 4 μm and immunostaining was done using a TechMate automated immunostainer (Dako, Ely, Cambridgeshire, United Kingdom) with 13 primary antibodies (Table 3). A standard 3′-diaminobenzidine peroxidase-conjugated streptavidin-biotin method was used for detection. Tumors and tissues with known staining patterns were used as positive immunostaining controls and normal tissues served as nontumor controls.

Evaluation of immunohistochemistry. A single pathologist (G.M.C.) scored all immunohistochemistry. Any cytoplasmic staining with the cytokeratins (CK) was scored as positive. Membranous staining was scored for HER2 according to the HercepTest (Dako) as follows: 0, no staining or faint incomplete staining in <10% cells; 1, faint incomplete staining in >10% cells; 2, weak to moderate complete staining in >10% cells; 3, strong complete staining in >10% cells. The percentage of tumor cells with unequivocal nuclear staining for ER, progesterone receptor (PR), p53, p27, Ki-67 (MIB-1), Mcm-2, and cyclin E was recorded semiquantitatively (0, no staining; 1, <10%; 2, 11-25%; 3, 26-50%; 4, 51-75%; 5, >75%). Cytoplasmic staining was scored for Bcl-2 and c-Myc and both the intensity of staining (0-4) and the percentage of positive cells were recorded. A cutoff value was applied to each marker to indicate positive or negative staining. The most appropriate cutoff was selected by testing the different values against outcome at 10 years using Cox regression analysis in the UBC and, where applicable, the Nottingham series (data not shown). This analysis supported a threshold of 10% for ER, PR, p53, p27, and Mcm-2 as reported previously (25–28); a score of 3+ for HER2 and 25% for both cyclin E and Ki-67 (MIB-1). For Bcl-2, there was little difference between the different measures of positivity (i.e., percentage of positive cells versus intensity of staining) and a cutoff value of 10% was used. For c-Myc, both cytoplasmic and perinuclear staining were scored and unequivocal moderate or strong staining in 25% cells was considered positive (normal epithelium rarely showed moderate or strong staining in >25% of cells). For all of the markers, the most parsimonious fit with outcome was seen when the binary scoring system (positive versus negative) was used.

Statistics. Association between categorical variables was assessed using Pearson's χ^2 test (29). Where appropriate, a χ^2 test for trend was used. A Cox proportional hazards model was used to examine the association between survival and putative prognostic variables (30). We included a term for study stratum in the regression models because the UBC series consisted of cases accrued onto four different trials. The proportional hazards assumption was tested using standard log-log plots. Initially, each variable was assessed in univariate analyses as a categorical variable. Where appropriate, the variable was also treated as continuous and the two models were compared using an appropriate likelihood ratio test. A hazard ratio (HR) and 95% confidence intervals (95% CI) were estimated for each variable. Multivariate analyses of variables and survival were done using Cox proportional hazards regression model in a backward stepwise manner until the most parsimonious fit was obtained and adjusted HRs and their 95% CIs were estimated. The fit of different models was evaluated using likelihood ratio tests. All *P*s are two sided unless otherwise stated.

Unsupervised hierarchical clustering algorithms were implemented to analyze multidimensional data using the program CLUSTER for continuous or ordinal data (http://rana.lbl.gov/EisenSoftware.htm) and STATA for binary data. Euclidean metrics were used to measure distance between the immunohistochemical scores when expressed as ordinal variables (i.e., five-tier scoring system). The simple matching binary similarity coefficient was used as the measure of distance for binary data. The distance (similarity) between the clusters was measured using complete linkage.

Results

Developmental study: analysis of 13 protein biomarkers in the UBC series

Pathologic associations. As expected, there were strong associations between many of the biomarkers and tumor grade and size. Increasing tumor grade showed a significant inverse association with expression of ER ($\chi^2 = 32$; $P < 0.0005$), Bcl-2 ($\chi^2 = 29$; $P < 0.0005$), and PR ($\chi^2 = 28$; $P < 0.0005$) and a significant positive association with that of MIB-1 ($\chi^2 = 9$; $P = 0.011$), Mcm-2 ($\chi^2 = 13$; $P = 0.002$), cyclin E ($\chi^2 = 8$; $P < 0.02$), and p53 ($\chi^2 = 25$; $P < 0.0005$). Four markers were

associated with increasing tumor size when size was expressed as a binary variable (<20 or ≥20 mm): MIB-1 (χ^2 = 5; P = 0.035), Mcm-2 (χ^2 = 11; P = 0.001), cyclin E (χ^2 = 5; P < 0.032), and p53 (P < 0.024). Smaller tumor size was associated with expression of ER (χ^2 = 9.3; P = 0.002), Bcl-2 (χ^2 = 4; P < 0.044), PR (χ^2 = 9; P < 0.003), and CK 8/18 (χ^2 = 7; P = 0.0009). Of the 13 markers, only Mcm-2 (χ^2 = 7; P = 0.11) and HER2 (χ^2 = 4.3; P = 0.04) were significantly associated with positive nodal status and c-Myc was associated with node-negative disease (χ^2 = 5; P = 0.03).

Survival analyses. Tumor size ≥20 mm, positive nodal status, and histologic grade were strongly associated with an adverse outcome at 10 years. Each increased the relative risk of poor outcome over 10 years by 34%, 45%, and 50% (grade 3 versus grade 1) respectively (Table 4). NPI was associated with a significant increase in the hazard of death (P_{trend} < 0.001). Eight markers (ER, PR, Bcl-2, cyclin E, p53, MIB-1, CK 5/6, and HER2) showed a significant association with survival in univariate analyses (Table 4).

Multivariate regression analysis was done that included these eight markers and the NPI. This initial multivariate analysis was based on 310 cases that had data for all markers and the NPI. Only the NPI, ER, and Bcl-2 remained in the final model (Table 5, model 1). The robustness of this model was then tested by repeating the regression analysis using only the NPI, Bcl-2, and ER, as there was a greater number of cases (n = 403) with complete data for the three variables (Table 5, model 2). Both the NPI and Bcl-2 remained in the final model but ER was no longer significant (HR, 0.79; P = 0.179). The effect of Bcl-2 on survival within each NPI group is shown in Fig. 1. Bcl-2-expressing tumors in the NPI moderate prognostic group (MPG) had a 52% reduction in the risk of death compared with Bcl-2-negative cases (HR, 0.48; 95% CI, 0.29-0.79; P = 0.004) and Bcl-2 expressors in the poor prognostic group (PPG) had a 41% reduction in the risk of death compared with Bcl-2-negative cases (HR, 0.59; 95% CI, 0.43-0.84; P = 0.003). There were insufficient cases with events to test the effect of Bcl-2 on NPI good prognostic group (GPG).

Given the reported association between ER and Bcl-2 (31, 32), we tested for interaction between ER and Bcl-2 expression. Compared with Bcl-2−/ER− cases, the HR (95% CI) was 0.87 (0.53-1.4) for Bcl-2−/ER+ (P = n.s.), 0.72 (0.55-9.4) for Bcl-2+/ER− (P = 0.02), and 0.40 (0.31-0.52) for Bcl-2+/ER+ (P < 0.001). The test for an interaction among the three groups, however, was not significant (P = 0.13), suggesting that the markers were more likely to have a multiplicative effect on the HR than an independent one.

An unsupervised hierarchical cluster algorithm was used to see if higher-order interactions between the markers that were not detected by multivariate regression analysis could be identified and to test the prognostic significance of such an interaction. Nine markers (ER, PR, Bcl-2, p53, cyclin E, MIB-1, Mcm-2, HER2, and CK 5/6) were used based on the strength of their association with outcome by univariate analysis. This panel distinguished seven subgroups of tumors within the cohort that were significantly associated with outcome (likelihood ratio χ^2 = 41.86; 6 *df*; P < 0.001; data not shown). However, the prognostic significance of this classifier was less than that obtained when Bcl-2 was used as an adjunct to the NPI in the same set of cases (likelihood ratio χ^2 = 47.52; 2 *df*; $P > \chi^2 < 0.001$).

Table 4. Univariate survival analysis of UBC series at 10 years

Variable	n*	HR (95% CI)	P
Tumor grade	727		
1		1.0	0.676
2		0.92 (0.61-1.38)	0.037
3		1.50 (1.02-2.21)	
Tumor size (mm)	854		
<20		1.0	0.016
≥20		1.34 (1.05-1.67)	
Nodal status	800		
Negative		1.0	0.005
Positive		1.45 (1.12-1.9)	
NPI	557		
GPG		1.0	0.231
MPG		1.53 (0.76-3.10)	0.011
PPG		2.51 (1.23-5.12)	<0.001
NPI as linear trend		1.62 (1.29-2.04)	
Cyclin E	651	1.95 (1.31-2.90)	0.001
p53	680	1.48 (1.19-1.84)	0.001
MIB-1	646	1.64 (1.29-2.10)	<0.001
Mcm-2	632	1.28 (1.0-1.67)	0.064
PR	840	0.55 (0.45-0.68)	<0.001
CK 5/6	130	1.58 (1.13-2.20)	0.007
CK17	130	0.84 (0.31-2.25)	0.726
CK 8/18	870	0.96 (0.73-1.26)	0.757
HER2	892	1.81 (1.45-2.30)	<0.001
ER	899	0.52 (0.43-0.63)	<0.001
p27	640	0.85 (0.66-1.10)	0.225
c-Myc	622	1.06 (0.81-1.30)	0.887
Bcl-2	728	0.53 (0.43-0.66)	<0.001

*Number of cases with data.

Table 5. Multivariate analysis of UBC series incorporating NPI as prognostic variable

Variables in final model	HR (95% CI)	P
Model 1* (n = 310)		
NPI	1.35 (1.16-1.56)	<0.0005
ER+	0.59 (0.39-0.88)	0.011
Bcl-2+	0.68 (0.46-0.99)	0.055
Model 2 (n = 403)†		
Bcl-2+	0.62 (0.44-0.87)	0.006
NPI	1.50 (1.16-1.56)	0.004

NOTE: n, number of cases with data.
*Initial model included cyclin E, p53, c-erbB-2, MIB-1, CK 5/6, ER, PR, Bcl-2, and NPI.
†Initial model included ER, Bcl-2, and NPI.

Validation study of Bcl-2 as a prognostic marker independent of NPI in the Nottingham series

Survival analyses. Based on the results of the UBC series, the Nottingham series was used to validate the prognostic

significance of Bcl-2. Data for ER and PR were also evaluated because ER remained prognostically independent of NPI in the first multivariate analysis of the UBC series (Table 5, model 1) and because of the accepted interdependence of ER and PR expression in breast cancer. Univariate analyses of survival (Table 6) revealed that grade, nodal status, tumor size, the NPI, and all three markers were significantly associated with outcome. The effect of increasing histologic grade was more marked in the Nottingham series (grade 3 versus grade 1: HR, 12.98; 95% CI, 5.73-29.38) than in the UBC series (grade 3 versus grade 1: HR, 1.50; 95% CI, 1.02-2.21), and as a result, the NPI was a more powerful indicator of outcome than it was in the UBC series. The effect of Bcl-2 positivity was similar to the UBC series with a 33% reduction in risk of death. There was also evidence of a dose effect for Bcl-2, with a better survival being observed with higher scores (data not shown). The effect of Bcl-2 expression on outcome was present for each of the NPI groups (Fig. 2): Bcl-2 distinguished two outcome groups within the GPG, MPG, and PPG. For all three markers (ER, PR, and BCL-2), the beneficial effect of positive expression was maximal in the first 59 months after diagnosis and waned with time (Table 7). Multivariate analysis confirmed the independent prognostic significance of Bcl-2 (P = 0.002) in the presence of the NPI (P±0.001). In the Nottingham series, PR (P = 0.004) also remained an independent predictor of outcome (Table 8).

Discussion

The NPI is one of the most widely used prognostic indices for patients with invasive breast carcinoma. It combines lymph node stage, tumor grade, and tumor size, which remain the strongest independent predictors of outcome, to give an individualized prognostic score for each patient. Cutoff points are applied to divide patients into GPG, MPG, and PPG that correlate strongly with survival (15 year-survival rate, 80%, 42%, and 13%; ref. 33). Since it was first developed, there have been many attempts to improve the prognostic power of the NPI, but most have met with limited success. Some have suggested modification of the variables used to determine the index (34, 35) or combined additional markers with it (1, 36–38) and others have used different statistical approaches (34). However, to date, very few markers have been validated as independent prognostic factors against the NPI in large series. Vascular invasion (1) and steroid hormone receptors have been shown to have a role largely limited to the GPG and PPG, respectively, but vascular invasion is difficult to assess; in both reports, these additional factors were examined in relative isolation. Smaller studies have reported that Bcl-2 (39), S-phase function, urokinase-type and plasminogen activator (38), steroid hormone receptor status (37), and Mcm-2 (40) had prognostic power independent of the NPI, but these findings need to be reproduced in larger studies and their value relative to other markers remains to be established.

In the developmental study presented here, Bcl-2 was the only marker from a panel of 13 protein biomarkers that could improve the prognostic power of the NPI. Overall, its expression reduced the likelihood of an adverse outcome at 10 years by 38% in the UBC series and could separate both MPG and PPG into two groups with significantly different outcomes. Bcl-2-negative tumors in the MPG had the same outcome at 10 years as Bcl-2-positive cases in the PPG (42%). Although the expression of nine of the markers when used together as a panel could identify prognostically distinct subgroups within the UBC series, this approach was less powerful than the use of Bcl-2 alone. The independent prognostic power of Bcl-2 was then validated in an independent large series of

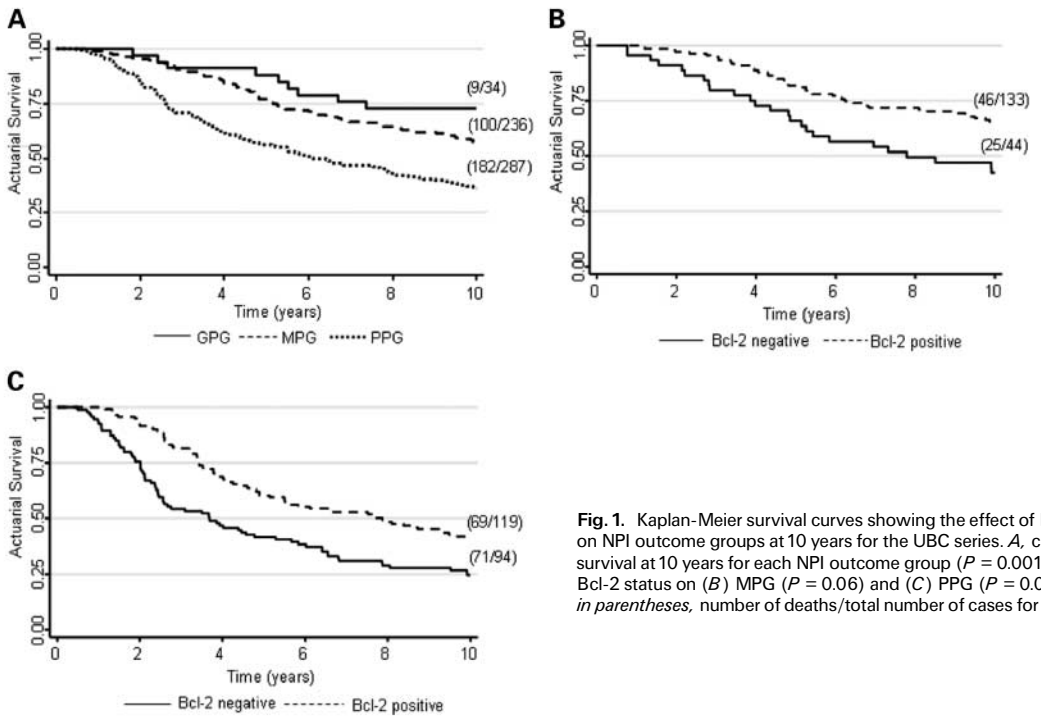

Fig. 1. Kaplan-Meier survival curves showing the effect of Bcl-2 status on NPI outcome groups at 10 years for the UBC series. *A,* cumulative survival at 10 years for each NPI outcome group (P = 0.001). Effect of Bcl-2 status on (*B*) MPG (P = 0.06) and (*C*) PPG (P = 0.003). *Number in parentheses,* number of deaths/total number of cases for each subgroup.

Table 6. Univariate analysis of survival for the Nottingham series

Variable	*n*	HR (95% CI)	*P*
Tumor grade			
2	1,914	3.77 (1.59-8.95)	0.003
3		12.98 (5.73-29.38)	<0.0005
Tumor size (mm)			
≥20	1,917	1.31 (1.21-1.41)	<0.0005
Nodal status			
1-3 nodes	1,912	1.84 (1.3-2.58)	<0.0005
> 4 nodes		7.59 (5.31-10.83)	<0.0005
NPI			
MPG	1,908	4.30 (2.49-7.42)	<0.0005
PPG		16.73 (9.62-29.07)	<0.0005
Bcl-2*			
Positive	983	0.67 (0.59-0.76)	<0.0005
ER			
Positive	1,788	0.31 (0.23-0.42)	<0.0005
PR			
Positive	1,764	0.33 (0.24-0.46)	<0.0005

NOTE: n, number of cases with data.
*The number of cores in which Bcl-2 could be scored was less than for ER and PR because Bcl-2 was evaluated on deeper levels from the tissue microarray where there was loss of many tumor cores. The survival of patients for which Bcl-2 data were available was no different to that for the whole series (data not shown).

cases where its prognostic effect was maximal in the first 5 years after the diagnosis of breast cancer.

The work reported here is the largest study yet to examine the prognostic role of Bcl-2 in breast cancer and the first to confirm it as a marker that is independent of, and additive to, the NPI. The effect of Bcl-2 was slightly smaller in the Nottingham series compared with the UBC series and it is possible that this difference will be explained by the characteristics of the two cohorts of patients. The latter consisted of cases accrued into four clinical trials between 1970 and 1990 and 51% of patients died from disease as a result of the high proportion of high-risk subsets (70% node-positive, 41% stage III). Hormonal therapy was routinely used for ER-positive patients (46% of the series) and all patients received either neoadjuvant or adjuvant chemotherapy. In contrast, the Nottingham series was a consecutive series in which ~90% of patients remained disease free. Sixty-four percent were node negative, 8% had stage III disease, and 70% were ER-positive. Given these differences, it is likely that the result in the Nottingham series is a more accurate reflection of the actual prognostic power of Bcl-2 in unselected patients. It was notable that the adverse effect of increasing histologic grade (and as a consequence the NPI score) was more dramatic in the Nottingham than the UBC series. This, again, is most likely due to the higher proportion of advanced cases in the UBC series and the fact that the effect of grade as an independent prognostic factor is greatest in node-negative disease. It should be noted that tumors in both series were graded by specialist breast pathologists. Not withstanding these differences in characteristics between the two series, the prognostic power of Bcl-2 was significant in both, strongly supporting the validity of the results.

One would predict that aberrations of the Bcl family of proteins might be prevalent in breast cancer given that impaired apoptosis is a crucial step in neoplastic progression and that the p53/Rb signaling pathway is dysregulated in most tumors. Bcl-2 belongs to the Bcl family of proteins that regulate apoptosis; whether a cell undergoes apoptosis or survives depends on the relative expression and dimerization status of the proapoptotic

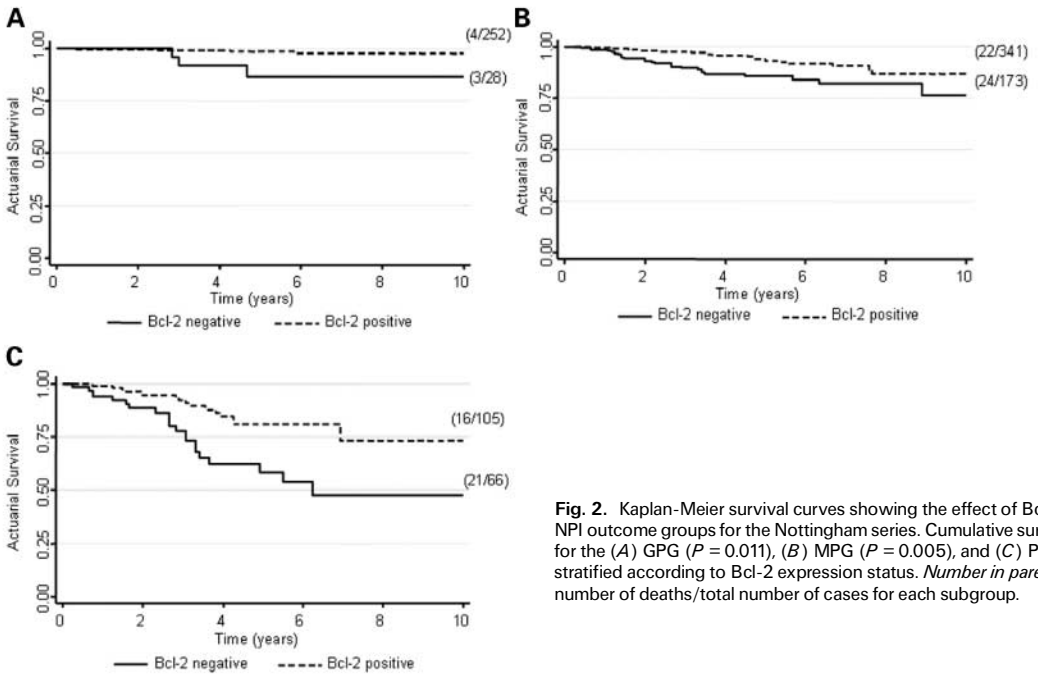

Fig. 2. Kaplan-Meier survival curves showing the effect of Bcl-2 status on NPI outcome groups for the Nottingham series. Cumulative survival at 10 years for the (*A*) GPG ($P = 0.011$), (*B*) MPG ($P = 0.005$), and (*C*) PPG ($P = 0.002$) stratified according to Bcl-2 expression status. *Number in parentheses,* number of deaths/total number of cases for each subgroup.

Table 7. Time-dependent effect of Bcl-2, ER, and PR

Marker	Period (mo)	HR (95% CI)	*P*	Likelihood ratio χ^2 (*P* > χ^2)
Bcl-2 (*n* = 997)	0-23	0.60 (0.47-0.78)	<0.0005	45.78 (<0.0005)
	24-59	0.64 (0.53-0.76)	<0.0005	
	>60	0.90 (0.66-1.22)	0.496	
ER (*n* = 1,812)	0-23	0.14 (0.74-0.25)	<0.0005	72.60 (<0.0005)
	24-59	0.36 (0.24-0.54)	<0.0005	
	>60	0.83 (0.40-1.76)	0.631	
PR (*n* = 1,789)	0-23	0.18 (0.10-0.36)	<0.0005	60.26 (<0.0005)
	24-59	0.32 (0.21-0.51)	<0.0005	
	>60	0.76 (0.38-1.51)	0.437	

NOTE: n, Number of cases with data.

(Bax, Bcl-xs, Bas, Bik/Nbk, Bid, and Bag-1) and antiapoptotic (Bcl-2, Bcl-XL, Bcl-w, A1, and Mcl-1) proteins. An increase in Bcl-2 shifts the balance in favor of cell survival. The tumorigenic potential of Bcl-2 has been shown in animal models (41, 42) and is supported by the finding of overexpression of Bcl-2 in a variety of solid organ tumors and in lymphomas (43, 44). In the latter, this results from chromosomal translocation and is associated with an adverse outcome. The mechanisms underlying Bcl-2 overexpression in other tumors and its significance are less certain. In the breast, Bcl-2 is expressed in normal glandular epithelium and is up-regulated by estrogen possibly as a result of direct transcriptional induction with negative regulation by p53-dependent mechanisms (31, 32, 45). In breast cancer, Bcl-2 expression is associated with markers of better differentiation (e.g., grade 1 lesions, which are ER-positive with low proliferative status, as we confirmed in this work; data not shown).

Most previous studies of Bcl-2 in breast cancer have also shown a favorable association between Bcl-2 positivity and outcome at least in univariate analysis. However, the majority of these have been small series in which very few markers were examined in parallel (e.g., ER, PR, and p53). Only two small studies (39, 46) have included the NPI in the analysis and only one (48) of the two larger studies (47, 48) showed a prognostic role for Bcl-2 expression that was maintained in multivariate analysis in node-positive disease.

Whether the prognostic role of Bcl-2 is consequent on its role in apoptosis or whether proposed nonapoptotic functions of Bcl-2 are somehow involved is unknown. Nonapoptotic functions have been described; interestingly, *in vitro* experiments have revealed that high levels of Bcl-2 can result in dramatic growth inhibition in different cell types (44). Indeed, a role in prolonging the cell cycle has been proposed (49–51).

Table 8. Multivariate analysis of survival for the Nottingham series

	HR (95% CI)	*P*
NPI	2.04 (1.67-2.51)	<0.001
PR+	0.42 (0.24-0.71)	0.002
Bcl-2+	0.83 (0.71-0.96)	0.018

NOTE: Likelihood ratio χ^2 = 97.05; *P* > χ^2 = 0.000; *n* cases = 928.

An interesting point that emerges from this work is that a very limited number of protein markers may be sufficient to improve prognostication. Expression array studies, in contrast, emphasize an approach that relies on the use of many genes to derive prognostic signatures, such as the 70-gene signature (11). This signature seemed more powerful than traditional pathologic variables (10), although these findings have not yet been widely validated, and some question their performance against the NPI (52). It must be noted that the relative importance of a single prognostic marker compared with a panel of marker(s) will depend on the choice and the nature of the markers that are included in the analysis. Studies that analyze gene expression cannot be compared directly with those in which protein expression is studied and this may explain why many of the markers included in this study have not emerged as prognostic candidates in expression array studies. Interestingly, *Bcl-2* has emerged as one of a panel of 16 informative genes that also includes *ER*, *PR*, *HER2*, and *Ki-67* whose expression can predict recurrence in tamoxifen-treated node-negative breast cancer (53) where transcript expression was evaluated by reverse transcription-PCR from fixed tumor material.

An obvious advantage of using immunohistochemistry is that it is relatively cheap and readily amenable to standardization in terms of methodology and interpretation, making it applicable for routine clinical use. However, immunohistochemistry is limited because an antibody may not detect all isoforms of a protein and this may be a source of contradictory reports about particular markers (54). In practice, a range of antibodies may need to be evaluated for each marker type. For example, we used both CK 17 and CK 5/6 to detect the basal phenotype and MIB-1 and Mcm-2 for assessment of proliferation.

In conclusion, our work provides convincing evidence that Bcl-2 can be used as an adjunct to the NPI to improve prognostication for an individual patient particularly in the first 5 years after a diagnosis of invasive breast cancer. A prospective study that includes Bcl-2 as part of a panel of potential prognostic and predictive markers is now needed.

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