ANTICANCER RESEARCH *24*: 473-482 (2004)

Expression and Prognostic Relevance of Mcl-1 in Breast Cancer

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Abstract. *Background: Bcl-2, an anti-apoptotic protein, is frequently associated with favourable prognosis in breast cancer. The potential role of mcl-1, another bcl-2 family member, in breast cancer has not yet been defined. Patients and Methods: This study examined the expression of mcl-1 and bcl-2 in 170 cases of invasive primary breast carcinoma, using reverse-transcriptase polymerase chain reaction and immunohistochemical analyses. Results: Expression of bcl-2 mRNA and protein were found to be favourably associated with outcome for patients, supporting a prognostic role for bcl-2 in breast cancer, whereas mcl-1 expression, at the mRNA or protein level, did not correlate with tumour size, grade, lymph node or ER status, age of patient at diagnosis, or disease outcome. Conclusion: As these analyses of mcl-1 expression may have co-detected mcl-1$_{S/\Delta TM}$ (a more recently identified, shorter variant, that may be pro-apoptotic) with the anti-apoptotic wild-type of mcl-1, it is possible that future studies may indicate some significant clinical correlations if the isoforms can be independently investigated.*

The bcl family of intracellular proteins plays a key role in regulating the survival and apoptosis of cells and tissues. Some of the family members (typified by bcl-2) protect against cell death, while others (*e.g.* bax) promote cell death by apoptosis (see reviews: (1,2)). Mcl-1 (myeloid cell leukemia factor-1), originally cloned as an early induction gene during the differentiation of the myeloid cell line, ML-1, is a 36 kDa anti-apoptotic member of the bcl-2 family (3). Mcl-1 expression is induced through anti-apoptotic cytokine-mediated pathways in polymorphonuclear neutrophils cells *via* interleukin and colony-stimulating factor pathways (4). It has been proposed that mcl-1 expression may be induced prior to bcl-2 expression, mediating rapid, *albeit* short-term, protection against cell death (5); the short half-life of mcl-1 (30 minutes to 3 hours) may confer on the cells an ability to respond rapidly to an apoptotic stimulus, until those with longer half-lives, such as bcl-2 (half-life approximately 10 hours) can, if needed, be recruited.

Mcl-1 expression has been investigated in a number of tumour types, but its functional significance in human cancers has not been conclusively established. Mcl-1 has been associated with poor prognosis in a study of 185 advanced ovarian tumours, with diffuse cytoplasmic staining, as opposed to peri-nuclear staining, associated with poor outcome for patients (6). Mcl-1 expression was also associated with poor prognosis in a study of 31 liver metastases from colorectal cancer (7) and the intensity of mcl-1 staining was found to be stronger in pulmonary lymphangioleiomyomatosis cells (from 9 patients) compared to normal vascular and bronchial smooth muscle cells (8). Increased expression of mcl-1 (and of bcl-2 and bcl-XL) has been associated with prostate cancer progression, as detected in 52/64 tumours (9). Surprisingly, a positive association was found between mcl-1 protein expression and high apoptotic index in a study of 15 large cell non-neuroendocrine lung carcinomas (10). Conflicting results from specific tumour types have

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Key Words: Apoptosis, bcl-2, breast cancer, disease/relapse-free survival, immunohistochemistry, mcl-1, overall survival, prognostic indicator, reverse-transcriptase polymerase chain reaction.

been reported. For example, in a study of 28 ovarian surface epithelial tumours, mcl-1 expression did not differ significantly between benign, borderline and malignant tumours (11). Shigemasa *et al.* (12) reported a study of 36 ovarian tumours where high levels of mcl-1 mRNA correlated with poor survival in stage III patients. Expression of mcl-1 protein indicated that diffuse expression in >50% of cells within a tumour associated significantly with poor survival rates. While mcl-1 overexpression was reported in anaplastic large cell lymphomas, inversely correlating with bcl-2 protein expression (15/18 bcl-2$^-$ were mcl-1$^+$; 8/16 bcl-2$^+$ were mcl-1$^-$), no significant association existed between mcl-1 expression and clinical features or with relapse-free survival (13). Mcl-1 protein expression detected in 75% (36/48) adenocarcinomas of the stomach showed no correlation with tumour histology or clinical stage (14). Peri-nuclear expression (in >10% of tumour cells) of mcl-1 in 70% of cervical tumours significantly associated favourably with overall survival (15).

Clinico-pathological investigations of the role of mcl-1 in breast cancer focusing on its importance as a prognostic factor have been limited and have generally been restricted to mcl-1 protein analysis only and have not included studies of mcl-1 mRNA. Analysis of 20 breast cancer biopsies, each containing normal, carcinoma *in situ* and invasive carcinoma, indicated that, although the intensity of mcl-1 staining was significantly lower in carcinoma cells than in normal epithelium, this was not the case when comparing carcinomas *in situ* with normal cells (16). Mcl-1 protein was found to be expressed in 68% (75/110) of cases in a study of primary invasive ductal breast carcinoma, where no patient had received endocrine or cytotoxic therapy. No correlation was identified between mcl-1 staining and apoptotic index, tumour grade, ER expression, or p53 expression (17). An association between mcl-1 expression and disease outcome was not investigated. In the study described here, we have investigated the expression of mcl-1 mRNA and protein in a retrospective series of primary breast cancers and investigated association with bcl-2 expression, clinicopathological factors, as well as disease/relapse-free survival (RFS) and overall survival (OS).

Materials and Methods

Patient selection. The study material was derived from 170 cases of primary breast cancer, on which clinical follow-up and pathologic material, including snap-frozen tissue, was available for analysis from the 1993-1997 files of St Vincent's University Hospital Pathology Department, Dublin, Ireland. The patients involved underwent potentially curative resection at the hospital. A number of clinical and pathologic parameters were abstracted from patients' charts including details on age, post–operative treatment and follow–up, tumour stage and hormonal analysis. Pathologic material was examined on each case by SK. Tumours were typed (18) and graded (19) as previously described. Staging was performed according to the TNM system of the UICC (20).

Reverse-transcriptase polymerase chain reaction. For RNA analyses, dissected tumours were homogenised, on ice, in 2 ml TriReagent (Sigma; Poole, England) and total RNA was subsequently isolated according to the manufacturer's instructions. First-strand cDNA was synthesised from 1 μg total RNA using oligo (dT) primer (Oswel; Southampton, England). Five μl cDNA was then amplified in a 50 μl PCR solution containing 1.5 mmol/l $MgCl_2$, 0.2 mmol/l deoxynucleotide triphosphates, 20 μmol/l oligonucleotide primers and 2.5 U *Taq* polymerase enzyme (Sigma). Forward and reverse primers for mcl-1 cDNA amplification used were - (forward): 5' TCTCTCG GTACCTTCGGG 3' and (reverse): 5' CTAT CTTATTAGAT ATGC 3' resulting in amplification of a 217 bp product. β-actin (forward): 5' TGGACATCCGCAAAGAC CTG TAC 3' and (reverse): 5' TCAGGAGGAGCAATGA TCTTGA 3' primers were used to co-amplify a 142 bp β-actin product. Amplification using bcl-2-specific primers - (forward): 5' TCATGT GTGTGGAGAGCGTCAA 3' and (reverse): 5' CTACTGC TTTAGTGAACCTTTTGC 3' resulted in amplification of a 306 bp product. A β-actin 383 bp region was co-amplified with bcl-2, as endogenous control, using the following primers, (forward): 5' GAAATCGTGCGTGACATTAAGGAGAAGGT 3' and (reverse): 5' TCAGGAGGAGCAATGATCTTGA 3'. The PCR cycle used was as follows: 94˚C for 2 min; 30 cycles of 94˚C for 30 sec, 53˚C (mcl-1) and 50˚C (bcl-2) for 30 sec, 72˚C for 30 sec; completion step of 72˚C for 5 min.

Immunohistochemistry. Formalin-fixed, paraffin-embedded tissue sections were immuno-stained for mcl-1 and bcl-2. Four-micron sections were dewaxed in xylene, rehydrated in alcohol and blocked for endogenous activity (3% H_2O_2 and normal rabbit serum). Antigen retrieval was carried out by pressure cooking in citrate buffer, pH 6.0. The sections were then incubated overnight at 4˚C with monoclonal antibody to mcl-1 (Dako Ltd; Cambridgeshire, UK; A3534) at dilution of 1:200, or for 20 min at room temperature at a dilution of 1/40 using anti-bcl-2 (Dako; M0887) antibody. Sections were washed in PBS pH 7.4 to remove unbound antisera. Bound antibody was detected using ABC detection kit (Vector Laboratories Ltd; Peterborough, UK) with DAB as a chromogen. Slides were then lightly counter-stained with Crazzi's haematoxylin. Human tonsil sections were used as positive controls and included in each batch of 20 slides. As a negative control, duplicate sections were stained without exposure to primary antibodies.

Evaluation of immunohistochemistry results. Mcl-1 and bcl-2 immunoreactivity was evaluated semi-quantitatively according to the percentage of cells demonstrating distinct immunohistochemical reaction, assessing at least 5 areas at 400X magnification and assigned a score as follows; 0 < 5%; 1=5-20%; 2=21-50%; 3=51-75%; 4 >75%. The intensity of immunoreactivity was scored as 1 (weak), 2 (moderate), or 3 (strong). The results were separately evaluated and statistically analysed. Two observers (SK, RP) separately scored the cases and agreed upon any discrepancies at a double-headed microscope.

Statistical analysis. Statistical (univariate and multivariate) analyses of the results were performed using the SPSS 10.1

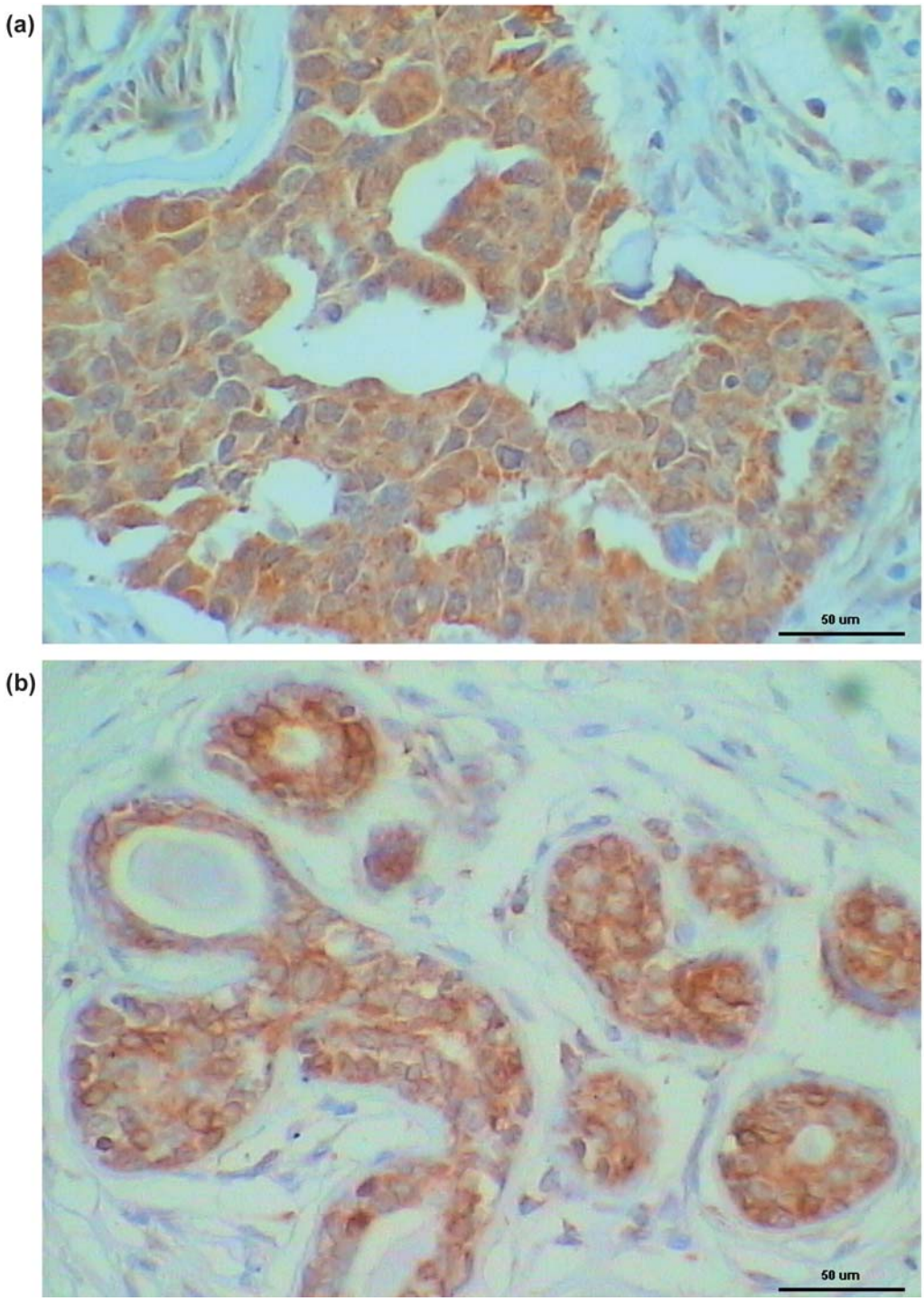

Figure 1. *Immunohistochemical staining for (a) mcl-1 and (b) bcl-2 protein expression. (400 X magnification; bar represents 50 μm).*

software package. Descriptive statistics were used to summarise patient characteristics and statistical analysis of the results was performed using Pearson's X^2 test to investigate relationships between reverse transcriptase-polymerase chain reaction, immunohistochemical and clinicopathological and histopathological findings. Kaplan-Meier survival curves were established and were subsequently checked using the log-rank, Breslow and Tarone-ware tests (*p*-values represent log-rank,

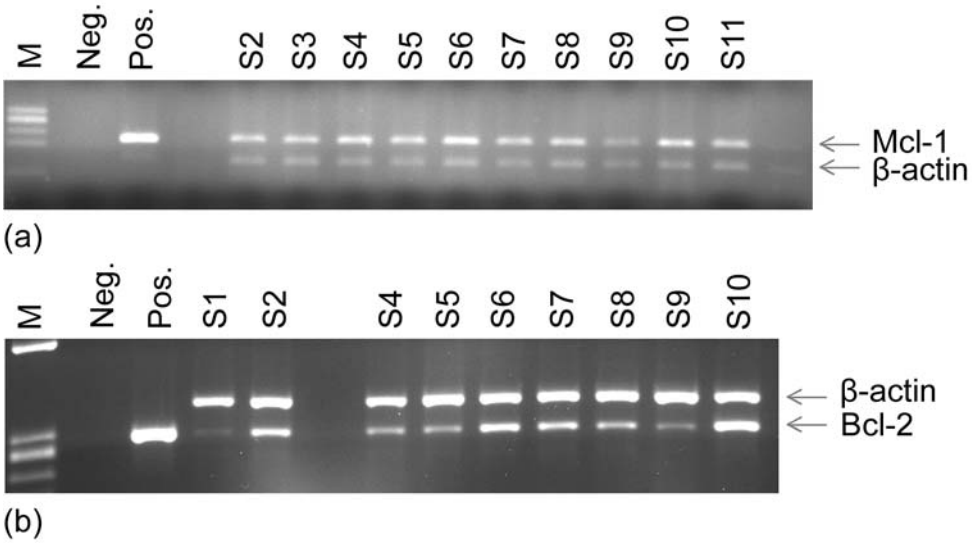

Figure 2. *Reverse-transcriptase polymerase chain reaction (RT-PCR) analysis of (a) mcl-1 and (b) bcl-2 in RNA extracted from 11 breast tumour biospies (S1-S11). β-actin was included as endogenous control, plasmid containing relevant cDNA as positive (Pos.) control, and H_2O as negative (Neg.) control. M indicates molecular weight markers.*

Table I. *Correlation between mcl-1 mRNA transcript and protein expression.*

Mcl-1 mRNA	Mcl-1 protein (> 0% *i.e.* present *vs*. absent)	Mcl-1 protein (> 20%)
Mcl-1 (-)	3/6 (50%)	3/6 (50%)
Mcl-1 (+)	55/85 (64.7%)	49/85 (57.6%)
P value	0.469	0.715

P value from X^2 analysis

Table II. *Correlation between Bcl-2 mRNA transcript and protein expression.*

Bcl-2 mRNA	Bcl-2 protein (> 0% *i.e.* present *vs*. absent)	Bcl-2 protein (> 20%)
Bcl-2 (-)	8/20 (40%)	8/20 (40%)
Bcl-2 (+)	54/68 (79.4%)	52/68 (76.5%)
P value	0.001	0.002

P value from X^2 analysis

unless otherwise indicated) to assess the prognostic significance of mcl-1 and bcl-2 expression in tumour cells. Multivariate survival analyses were performed using the Cox regression backward stepwise likelihood ratio. The data was censored at 5 years for multivariate analysis. A value of $p<0.05$ was considered statistically significant.

Results

Patient characteristics. The patients were aged between 31 years and 90 years at the time of diagnosis (mean age=58.7 years). Fifty-one women were less than 50 years and 119 women were 50 years, or older, at diagnosis. The size of the tumours varied between 0.6 cm and 9.0 cm (mean=2.86 cm). Thirty-two tumours were T1 (<2cm) in maximal dimension; 130 tumours were T2 (2-5cm) and 8 tumours were T3 (>5cm). One hundred and thirty-seven tumours were invasive ductal carcinoma, 25 were invasive lobular and 8 were tumours of special type (2 tubular and 6 mucinous).

Eighteen tumours were grade 1; 72 were grade 2 and 80 were grade 3. One hundred and ten tumours were oestrogen receptor-positive and 52 were oestrogen receptor-negative (oestrogen receptor status was determined by Enzyme Immuno-Assay (EIA). A positive result was defined as more than 200fmol/g protein.). Oestrogen receptor status was not available for 8 patients. Eighty tumours had no axillary metastases and 90 tumours had metastasised to axillary lymph nodes.

One hundred and three women were treated with post-operative tamoxifen, 53 did not receive tamoxifen. Eighty-five patients were treated with adjuvant systemic chemotherapy (CMF ± adriamycin). Seventy-two patients did not receive chemotherapy. Details regarding tamoxifen

Table III. *Univariate Cox analysis.*

Characteristics	Overall Survival (OS)	Relapse-Free Survival (RFS)
	P	*P*
Age (<50 *vs*. ≥50 yrs.)	0.550	0.701
Tumour size (<2.8 *vs*. ≥2.8 cm)	0.016*	0.009*
Lymph node metastasis (Negative *vs*. positive)	<0.0001*	<0.0001*
Histology grade (I & II *vs*. III)	<0.0001*	<0.0001*
Histology type (IDC *vs*. ILC *vs*. special)	0.057	0.579
ER status (Negative *vs*. positive)	0.070	0.032*
Mcl-1 Protein (absent *vs*. present)	0.243	0.993
Mcl-1 Protein (≤20% *vs*. >20%)	0.426	0.878
Bcl-2 mRNA (absent *vs*. present)	0.028*	0.025*
Bcl-2 Protein (absent *vs*. present)	0.069	0.066
Bcl-2 Protein (≤20% *vs*. >20%)	0.034*	0.016*

* significant parameter; mean size (2.8 cm) was used as cut-off; grade I & II were grouped together *versus* grade III; IDC=invasive ductal carcinoma; ILC=invasive lobular carcinoma.

Table IV. *Correlation between clinicopathological factors and expression of bcl-2 mRNA in breast carcinoma.*

Characteristics	Bcl-2 mRNA n	Bcl-2 (%)	*P*
Age (yr.)			
<50	15/21	71.4	0.464
≥50	53/67	79.1	
Tumour size			
T1 (<2 cm)	12/14	85.7	
T2 (2-5 cm)	54/70	77.1	0.323
T3 (> 5cm)	2/4	50.0	
Lymph node metastasis			
Negative	34/39	87.2	0.048*
Positive	34/49	69.4	
Histology grade			
I	8/8	100.0	
II	22/31	71.0	0.217
III	38/49	77.6	
Histology type			
IDC	53/69	76.8	
ILC	12/15	80.0	0.959
Special	3/4	75.0	
ER status			
Negative	18/29	62.1	0.012*
Positive	49/57	86.0	
Adjuvant chemotherapy			
No	29/38	76.3	0.843
Yes	32/43	74.4	
Tamoxifen			
No	14/21	66.7	0.286
Yes	47/60	78.3	

P values from X^2 analyses; * indicates significant parameter; IDC=invasive ductal carcinoma; ILC=invasive lobular carcinoma.

and systemic chemotherapy were not available for 14 and 13 patients, respectively. Maximal follow-up was 3165 days with a mean follow-up of 1805 days.

Immunohistochemical analysis. Specific staining for mcl-1 was observed in tumour cells in 68.2% (116/170) of cases, with mcl-1 localised in the cytoplasmic region of the cells (Figure 1a illustrates a typical example). Fifty-four tumours (31.8 %) did not express mcl-1. The breakdown of the distribution of mcl-1 in tumours was as follows: 7.7 % score 1; 23.3 % score 2; 20.7 % score 3; and 48.3 % score 4. Weak staining was observed 24.1% (28/116) cases, moderate staining in 41.4%, and 34.5% stained strongly. The percentage positivity and intensity of mcl-1 staining were found to be strongly associated ($p<0.001$).

Bcl-2 protein was also expressed in the cytoplasm and it was detected in 70.6% (120/170) tumours (see Figure 1b). The breakdown of the distribution of bcl-2 in tumours was as follows: 5 % score 1; 17.5 % score 2; 27.5 % score 3; and 50 % score 4.

Relationship between expression of mcl-1 and bcl-2 mRNAs and proteins. Levels of mRNAs encoding mcl-1 and bcl-2 were assessed, by RT-PCR, in a random selection of 53.5 % (91/170) and 51.8 % (88/170) of the breast tumour biopsies, respectively. Results indicating amplification of these mRNAs (described as positive or negative) in 11 specimens are shown in Figure 2, as representative results. Amplification of β-actin (142 bp) as endogenous control, in all cases, indicated that the mRNA was of sufficient quality and that the RT and PCR reactions were conducted successfully. Mcl-1 mRNA was detected in 93.4 % (85/91) but was not detected in 6.6 % (6/91) of cases. Mcl-1 mRNA detection did not correlate with mcl-1 protein expression, when analysed as protein presence or absence ($p=0.469$) or protein expression in >20% of the tumour cells ($p=0.715$)

Table V. *Correlation between clinicopathological factors and expression of mcl-1 and bcl-2 protein in breast carcinoma.*

Characteristics	Mcl-1						Bcl-2					
	No. of cases (>0 %)	mcl-1 (%)	*P*	No. of cases (>20 %)	mcl-1 (%)	*P*	No. of cases (>0 %)	bcl-2 (%)	*P*	No. of cases (>20 %)	bcl-2 (%)	*P*
Age (yr.)												
<50	36/ 51	70.6	0.666	33/51	64.7	0.755	37/51	72.5	0.713	34/51	66.7	0.943
≥50	80/119	67.2		74/119	62.2		83/119	69.7		80/119	67.2	
Tumour size												
T1 (<2 cm)	24/32	75.0		22/32	68.8		24/32	75.0		23/32	71.9	
T2 (2-5 cm)	86/130	66.2	0.576	80/130	61.5	0.751	92/130	70.8	0.380	87/130	66.9	0.499
T3 (> 5cm)	6/8	75.0		5/8	62.5		4/8	50.0		4/8	50.0	
Lymph node metastasis												
Negative	52/80	65.0	0.393	50/80	62.5	0.911	57/80	71.3	0.858	55/80	68.8	0.658
Positive	64/90	71.1		57/90	63.3		63/90	70.0		59/90	65.6	
Histology grade												
I	11/18	61.1		11/18	61.1		14/18	77.8		13/18	72.2	
II	50/72	69.4	0.787	46/72	63.9	0.970	54/72	75.0	0.312	52/72	72.2	0.315
III	55/80	68.8		50/80	62.5		52/80	65.0		49/80	61.3	
Histology type												
IDC	99/137	72.3		93/137	67.9		94/137	68.6		90/137	65.7	
ILC	13/25	52.0	0.071	10/25	40.0	0.022*	19/25	76.0	0.425	17/25	68.0	0.441
Special	4/8	50.0		4/8	50.0		7/8	87.5		7/8	87.5	
ER status												
Negative	36/52	69.2	0.986	31/52	59.6	0.471	24/52	46.2	<0.001*	22/52	42.3	<0.001*
Positive	76/110	69.1		72/110	65.5		94/110	85.5		90/110	81.8	
Adjuvant chemotherapy												
No	49/72	68.1	0.981	47/72	65.3	0.596	52/72	72.2	0.822	50/72	69.4	0.749
Yes	58/85	68.2		52/85	61.2		60/85	70.6		57/85	67.1	
Tamoxifen												
No	36/53	67.9	0.898	34/53	64.2	0.912	35/53	66.0	0.312	34/53	64.2	0.466
Yes	71/103	68.9		67/103	65.0		76/103	73.8		72/103	69.9	

P values from X^2 analyses; * indicates significant parameter; IDC=invasive ductal carcinoma; ILC=invasive lobular carcinoma.

Table VI. *Multivariate Cox regression backward stepwise likelihood ratio.*

Characteristics	Overall Survival (OS)	Relapse-Free Survival (RFS)
	P	*P*
Lymph node metastasis (Negative *vs*. positive)	<0.0001	0.001
Histology grade (I & II *vs*. III)	0.001	0.019
ER status (Negative *vs*. positive)	0.012	0.017

Parameters included in the multivariate analysis were age, tumour size, tumour grade, tumour type, lymph node status, treatment with chemotherapy, treatment with tamoxifen, as well as mcl-1 and bcl-2 expression. Mean size (2.8 cm) was used as cut-off; grade I & II were grouped together *versus* grade III. This Table summarises significant factors.

(Table I). Bcl-2 mRNA was amplified in 77.3% (68/88) of biopsies, but was not detectable in the remaining 22.7%. Unlike mcl-1, bcl-2 mRNA detection correlated with bcl-2 protein expression, when analysed as protein presence or absence ($p=0.001$) and protein expression in >20% of the tumour cells ($p=0.002$) (Table II). No significant association was found between expression of mcl-1 and bcl-2 mRNA ($p=0.210$), mcl-1 and bcl-2 protein presence (*versus* absence) ($p=0.177$), or mcl-1 and bcl-2 expression in >20% tumour cells ($p=0.205$).

Prognostic analysis of disease-free survival. To evaluate the prognostic relevance of mcl-1 and bcl-2 expression at diagnosis, mcl-1 (protein) and bcl-2 (mRNA and protein) were analysed in relation to RFS and OS. As the majority (93.4%) of tumours were found to express mcl-1 mRNA, statistical evaluation of this was not included. By Cox

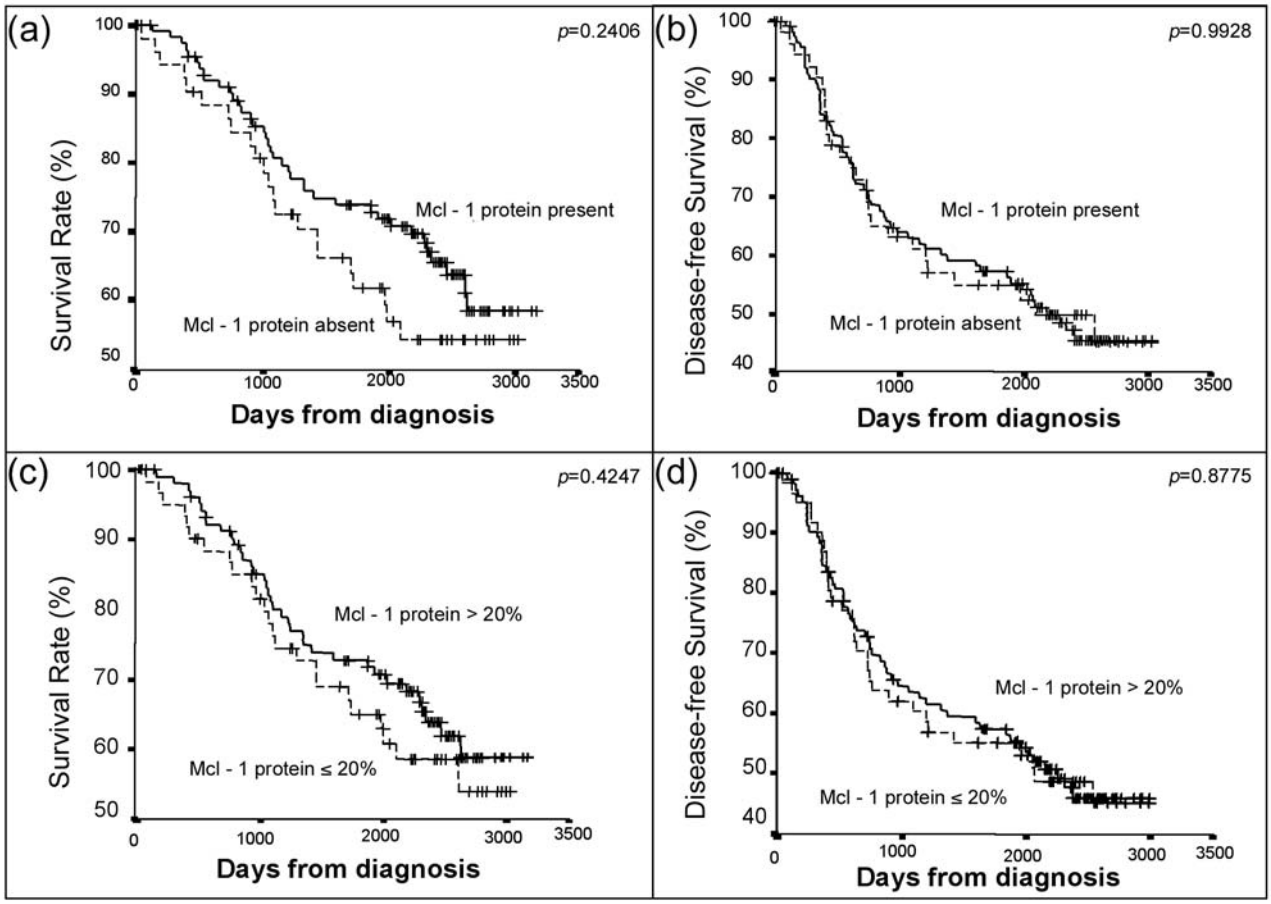

Figure 3. *Kaplan-Meier survival curves for mcl-1 protein presence or absence ((a) & (b)), and protein expression in >20% tumour cells (versus ≤20%) ((c) & (d)) for OS and RFS, respectively, indicates no association between mcl-1 expression and OS and RFS. (p=log-rank value).*

univariate analysis for RFS (Table III), bcl-2 mRNA ($p=0.025$) and protein expression in >20% of tumour cells ($p=0.016$) were associated favourably with RFS. Similarly, grade ($p<0.0001$), ER status ($p=0.032$), nodal status ($p<0.0001$) and tumour size ($p=0.009$) were significantly related to RFS, whereas mcl-1 protein expression, age and histological type of tumour were not. Using Chi-squared analysis, a significant association was detected between expression of mcl-1 in >20% tumour cells and tumour type ($p=0.022$) (Table V). Mcl-1 expression was not associated significantly with the age of the patients at diagnosis, tumour size, nodal status, ER status or treatment (whether tamoxifen or chemotherapy). By Chi-squared analysis, bcl-2 mRNA expression was found to correlate significantly with lymph node status ($p=0.048$), and bcl-2 mRNA ($p=0.012$) and protein expression ($p<0.001$) associated significantly with ER status. No significant association between mcl-1 or bcl-2 was found with any other clinicopathological factor analysed (Tables IV and V). Similarly, no significant association was found between mcl-1 staining intensity and RFS ($p=0.9169$).

From multivariate analysis, the most important prognostic factors for RFS were found to be negative lymph node status ($p=0.001$), ER status ($p=0.017$) and tumour grade ($p=0.019$) (Table VI). Whereas Kaplan-Meier analysis for mcl-1 protein showed no association with RFS (Figure 3), the Kaplan-Meier survival curves (see Figure 4) demonstrated better prognosis from expression of bcl-2 mRNA ($p=0.0218$) and bcl-2 protein in >20% tumour cells ($p=0.0151$).

Prognostic analysis of overall survival. Analysis of overall survival, using Cox regression univariate analysis, indicated that the important prognostic factors were tumour size ($p=0.016$), tumour grade ($p<0.0001$), lymph node status

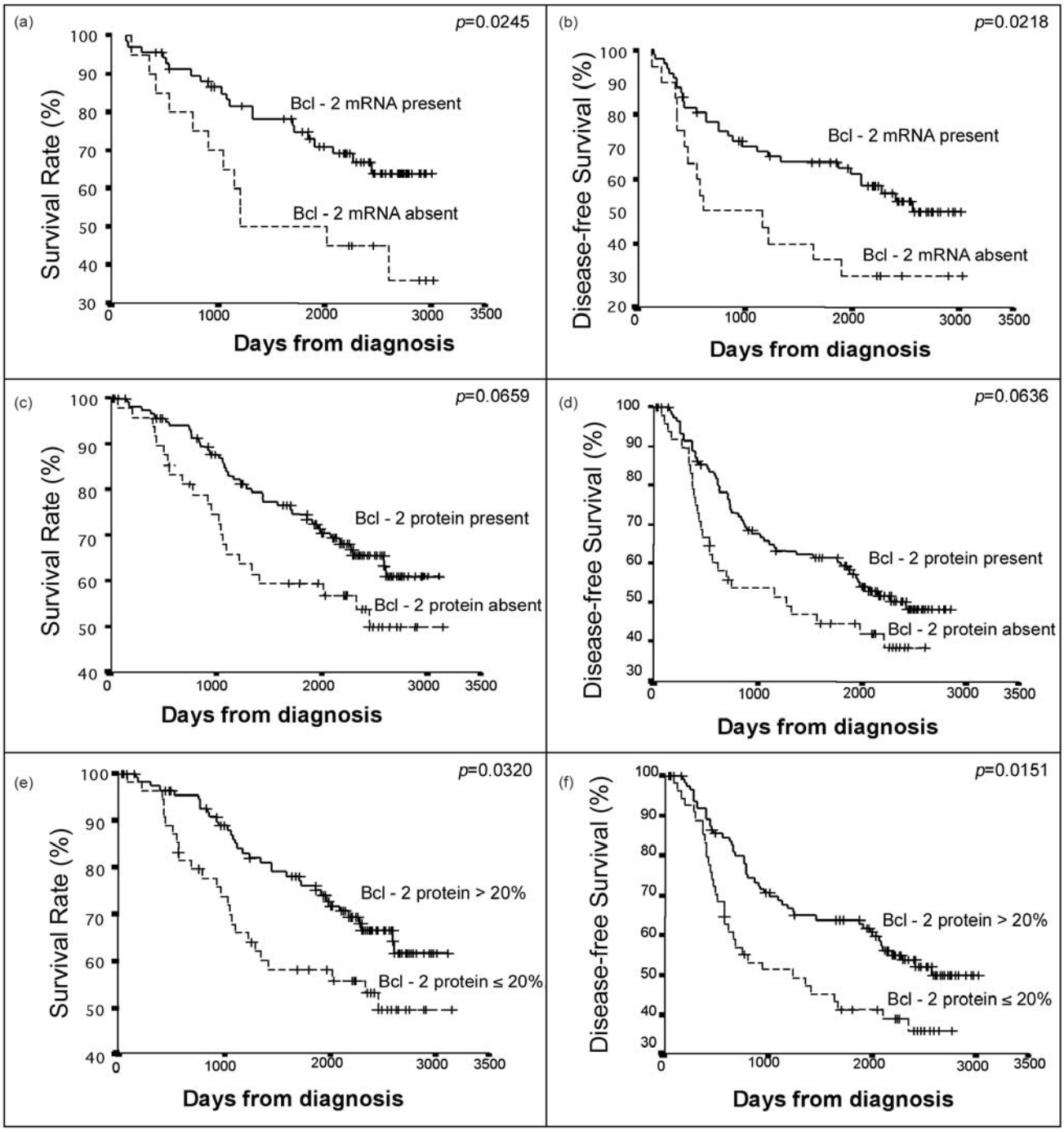

Figure 4. *Kaplan-Meier survival curves for breast carcinoma categorised according to bcl-2 expression indicating a favourable association between bcl-2 mRNA (a & b) and protein expression in >20% tumour cells (e & f), respectively, with both RFS and OS. (p=log-rank value).*

($p<0.0001$) and bcl-2 mRNA ($p=0.028$) and protein expression in >20% of tumour cells ($p=0.034$) (Table III). The favourable association with bcl-2 expression (see Figure 4) and lack of association with mcl-1 (Figure 3) was supported by Kaplan-Meier analysis. No significant association was found between mcl-1 staining intensity and OS (P=0.6571). As for RFS, multivariate analysis indicated that the most important prognostic factors for overall survival were negative lymph node status ($p<0.0001$), ER status ($p=0.012$) and lower (I & II *versus* III) tumour grades ($p=0.001$) (Table VI).

To further investigate the relevance of mcl-1 expression for this group of people with breast cancer, Chi-squared and Kaplan-Meier analyses was performed on sub-populations of patients who had received chemotherapy and those who had not received this adjuvant therapy. No significant correlation was found between mcl-1 protein expression with RFS or overall survival within either of these sub-groups.

Discussion

This study investigated, for the first time, the relevance of mcl-1 expression, at both the mRNA and protein levels (considering both the percentage of tumour cells expressing mcl-1 and the intensity of staining), to outcome for patients with breast cancer. Unlike the previous study of mcl-1 expression in chemotherapy and tamoxifen naïve patients (17), this retrospective study investigated the relevance of mcl-1 in a group of 170 patients where treatment regime and outcome were known and so could be considered over the mean follow-up period of 1805 days (maximum follow-up of more than 8.5 years). Expression of bcl-2, another bcl family member, previously shown to be associated with favourable outcome in breast cancer (21, 22), was also investigated.

The majority of breast tumours analysed expressed mcl-1 (68.2%) and bcl-2 (70.6%) proteins, with both proteins detected in 47% of cases; 40% of biopsies expressed both mcl-1 and bcl-2 in >20% of tumour cells. Mcl-1 and bcl-2 proteins were both undetectable in only approximately 8% of cases. The data presented here indicate that bcl-2 mRNA and protein expression are both significant prognostic factors associated with favourable outcome in terms of both RFS and OS for this group of breast cancer patients. Based on previous reports of bcl-2 expression in breast tumours, this suggests that the group of patients studied represented a "typical" breast cancer population. As in the analysis of pre-treatment breast tumours reported by Rochaix *et al.* (17), 68% of tumours included in this study expressed mcl-1 protein. Unlike bcl-2, however, expression of neither mcl-1 mRNA or protein (presence or intensity) were found to be associated with RFS or OS and expression of mcl-1 mRNA and protein were not significantly associated, although the expression levels (percentage of positive tumour cells) and intensity of expression were strongly associated. Similarly to studies of breast biopsies previously reported (17), mcl-1 protein expression was not associated with tumour grade or ER status. No association was found with the age of the patients at diagnosis, lymph node status or tumour size, although a significant association was found between expression of mcl-1 (in >20% of tumour cells) and histological type of tumour, with mcl-1 protein expression most frequently detected in invasive ductal carcinomas. Further analysis of mcl-1 expression in sub-populations of patients – including those who received adjuvant chemotherapy compared to those who did not receive chemotherapy; those with lymph node metastasis compared to those with no spread to nodes; those with grade III tumours compared to those with grades I or II – emphasised that mcl-1 cannot be considered a prognostic factor, as no significant correlation was found with RFS or OS.

An alternative splice variant of mcl-1, termed mcl-$1_{S/\Delta TM}$, resulting from excision of exon 2 and so expression of a death-inducing gene product when introduced into cultured cells, has been reported (23). The first 229 amino acids of this 271 amino acid pro-apoptotic isoform are identical to those of the anti-apoptotic form of mcl-1, but the remaining 42 amino acids vary, due to a shift in reading frame in the C-terminals of mcl-1 and mcl-$1_{S/\Delta TM}$. The majority of, if not all, studies of mcl-1 previously reported (7, 12, 13, 17, 24), as well as the study reported here, co-detect both mcl-1 splice variants. The fact that the levels of the alternate splice variant, mcl-$1_{S/\Delta TM}$, were lower in abundance than those of full-length wild-type mcl-1 in the cell lines tested (23) may indicate that the short variant may be less relevant *in vivo*. It must be considered, however, that lack of detectable prognostic significance for mcl-1 may be due to limitations of available detection methods. Further studies of clinical biopsies using primers and antibodies developed specifically for mcl-1 and mcl-$1_{S/\Delta TM}$, respectively, may indicate the relevance of the alternate splice forms of mcl-1 *in vivo* and may help to explain why, unlike bcl-2 mRNA and protein expression, expression of mcl-1 mRNA and protein in this group of breast biopsies were not significantly associated.

In conclusion, in this study of breast tumours the main prognostic indicators of RFS and OS were found to be lymph node metastasis, ER status and tumour grade. Bcl-2 mRNA and protein expression were favourably associated with outcome for patients, whereas expression of mcl-1 mRNA or protein apparently is not a significant prognostic indicator of outcome. Further studies independently investigating expression of the mcl-1 splice variants, when selective antibodies become available, may elucidate a possible role for mcl-1 in apoptosis control in breast cancer.

Acknowledgements

This work was supported by grants from The Health Research Board of Ireland, Enterprise Ireland, Cancer Research Ireland, The Irish Higher Education Authority's PRTLI program and Research Foundation, Royal Victoria Eye and Ear Hospital.

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Received September 17, 2003
Accepted January 5, 2004