Automated and quantitative immunocytochemical assays of Bcl-2 protein in breast carcinomas

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Summary Expression of the bcl-2 gene was investigated in 218 human breast carcinomas by immunohistochemical analysis. Immunodetections were assessed using (1) frozen sections, (2) documented commercially available monoclonal antibody (bcl-2/124, Dako), (3) automation of immunoperoxidase technique (Ventana) and (4) quantitative evaluation of results by image analysis (SAMBa) and statistical analysis of quantitative data (BMDP software). Bcl-2 protein expression was correlated with current prognostic indicators and with molecular markers detected by the same procedure as for Bcl-2. It was shown that Bcl-2 expression is not related to patients' age, tumour size and type or lymph node status, but an inverse relationship was observed between Bcl-2 and tumour grade (P < 0.0001). An inverse relationship was also observed between Bcl-2 expression and p53 (P < 0.0001), Ki67/MIB1 antigen- (P = 0.0012), and P-gp- (P = 0.002) positive immunoreactions. In contrast, anti-Bcl-2 positive reaction was significantly associated with ER-positive (P < 0.001) and with ER/PR-positive or ER/PR/pS2-positive immunoreactions (P < 0.005). Bcl-2 expression was independent of CD31 and cathepsin D expression. Thus, Bcl-2 protein, thought to be antiapoptotic, exhibits paradoxical expression in human breast carcinomas. It is strongly detected in low-grade tumours (well-differentiated) with low (MIB1) growth fraction, but is independent of the tumour progression (size, node status, CD31, and cathepsin D). Bcl-2 acting on apoptosis is related to p53 gene abnormalities in breast carcinomas. Bcl-2 protein expression may also be involved in response to endocrine therapy (associated to ER/PR/pS2 positive immunoreactions) and probably with chemoresistance mechanisms (inverse relationship with P-gp).

Keywords: Bcl-2 protein; automated and quantitative immunohistochemistry; breast carcinoma

Bcl-2 gene abnormality was first reported in non-Hodgkin’s lymphomas (Tsujimoto et al, 1985), in which t(14;18) chromosomal translocation results in an inappropriately high level of bcl-2 gene expression (Korsmeyer, 1992). The protein encoded by this gene has been shown to contribute to malignant cell expansion by prolonging cell survival (Vaux et al, 1988) and delaying or blocking programmed cell death/apoptosis (Hockenbery et al, 1990; Siegel et al, 1992). The human Bcl-2 protein is an intracellular membrane protein with a molecular mass of 24 kDa (Chen-Levy et al, 1989, 1990). It resides in the nuclear envelope, endoplasmic reticulum and outer mitochondrial membranes (Krajewski et al, 1993) with a non-uniform distribution suggestive of participation in protein complexes (Krajewski et al, 1993).

Apoptosis is an active form of cell death (Wyllie et al, 1987), requiring activation of endonucleases inducing the degradation of nuclear DNA of the cell into oligonucleosome-length fragments. Apoptosis plays an important role in normal physiological conditions, but in proliferative cells and tumours it may also be the target for chemotherapeutic drugs that can kill cancer cells by activating biochemical pathways leading to programmed cell death (Eastman et al, 1990). Drugs may act on the apoptotic process by inhibition of antiapoptotic factors up-regulated in malignant tumours. Chemoresistance may result from antiapoptotic protection from cell death and DNA fragmentation induced by antineoplastic drugs (Miyashita, 1992, 1993). In this regard high levels of Bcl-2 protein have been shown to protect lymphoid cells from antineoplastic drugs (Miyashita, 1992, 1993). In leukaemic cells, it can inhibit apoptosis induced by glucocorticoids (Alnemri et al, 1992) and 5-fluorodeoxyuridine or taxol (Fisher et al, 1993). Thus, detection of this protein in tissue may be informative for monitoring the therapy of patients with cancer. However, the true significance of Bcl-2 protein expression in tumours still remains to be investigated accurately, particularly in human breast carcinomas.

In breast carcinomas a high fraction of Bcl-2-positive cells correlates with good prognostic indicators (Silvestrini et al, 1994), longer survival in node-positive patients (Hellemans et al, 1995), and oestrogen receptor status (ER) (Alnemri et al, 1992; Johnston et al, 1994; Silvestrini et al, 1994; Hellemans et al, 1995; Baba et al, 1996). In addition, a highly significant relationship has been observed between the response to endocrine therapy and the presence of Bcl-2 protein in the tumour (Gee et al, 1994). Bcl-2 protein expression has been shown to be enhanced in ER-positive tumours after tamoxifen in association with reduced cell proliferation (Johnston et al, 1994). These results in breast carcinomas are somewhat paradoxical as it would be expected that Bcl-2 protein would counteract the tumour-inhibitory effect of endocrine therapy and that it would be down-regulated in tumours with good prognosis, with an inverse relationship with survival rates, as it is thought to prevent programmed cell death.

Only a few recent studies screening Bcl-2 protein expression in breast carcinomas have been reported (Silvestrini et al, 1994; Hellemans et al, 1995; Johnston et al, 1994; Baba et al, 1996), and in these reports methods of investigation were different. When

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immunohistochemistry was used to detect Bcl-2 protein in tissue sections from the tumours, samples were either fixed and paraffin embedded (Johnston et al, 1994; Silvestrini et al, 1994; Helleman et al, 1995; Baba et al, 1996) or frozen (Gee et al, 1994). Antibodies directed against Bcl-2 protein were also different and variably diluted. Results were evaluated by semiquantitative analysis, which relies on the observers' subjectivity, with variable and arbitrary 'cut-off' points (Johnston et al, 1994; Helleman et al, 1995). The rates of Bcl-2 protein-positive tumours were highly variable, ranging from 32% to 75% (Gee et al, 1994; Johnston et al, 1994; Silvestrini et al, 1994; Helleman et al, 1995; Baba et al, 1996). As already mentioned (Johnston et al, 1994), validation of statistical analysis is difficult because of the lack of standardization of immunohistochemical assays and therefore practical conclusions, particularly with regard to the clinical significance of Bcl-2 expression in tissue, may not be clearly drawn from the literature survey.

In the present study, we have evaluated Bcl-2 protein expression in 218 breast carcinomas using immunohistochemical assays assessed in optimal technical conditions. Bcl-2 expression was detected (1) on frozen sections (with no risk of antigen damage owing to fixation and heating during paraffin embedding), (2) using a well-documented commercially available antibody (Dako/Bcl-2, 124), (3) by automated immunodetection (Ventana device) more reproducible than manual procedures and (4) by quantitative analysis of the results by densitometry of digitized coloured microscopic images (image analyser SAMBA). Bcl-2 expression in tumours was correlated with histological prognostic indicators and with expression of proteins also endowed with prognostic significance, such as growth fraction (detected by MIB1), p53, CD-31 (angiogenesis), cathepsin D (extracellular matrix protease) and hormone receptors and P-gp (multidrug resistance) evaluated according to the same procedure.

**MATERIAL AND METHODS**

**Source of tissue samples**

The specimens were surgically obtained from 218 patients with breast carcinomas from January 1993 to May 1994. Mean age was 56.7 years (range 32–83 years). For all patients surgical resection was the primary treatment and none received irradiation or chemotherapy preoperatively. Surgical specimens were fixed in Bouin's fixative, paraffin embedded and stained with haematoxylin, eosin and safranin for routine microscopic diagnosis. Samples for immunodetection were taken, by pathologists, from the representative cancerous lesions, in the same area as the sample used for the intraoperative microscopic diagnostic assessed on frozen sections. Tissue samples for immunodetections were promptly dipped in liquid nitrogen and stored frozen at –80°C in the tumour library of our laboratory.

**Histopathological features**

Tumour size ranged from 4 to 80 mm (mean = 16.8, s.d. = 11.95, median = 15 mm). In 90% of patients (198/218), axillary lymph node excision was performed; 62% (n = 124) were node negative and 38% (n = 75) were node positive.

Tumours were in situ in 17/218 cases (8%). Invasive ductal carcinomas accounted for 66% of cases (143/218), lobular carcinomas for 17% (38/218) and invasive carcinomas of other types for 9% (20/218).

Tumours were graded according to the Bloom grading system (SBR) (Bloom, 1957). Grade I tumours accounted for 22% (45/201), grade II tumours for 54% (108/201) and grade III tumours for 24% (48/201). Tumours were also graded according to a modified Bloom grading system (Le Doussal et al, 1989) into five grades: 8% of grade I, 30% of grade II, 31% of grade III, 27% of grade IV and 4% of grade V. Tumours were also ranked according to the Nottingham prognostic index (NPI) (Galea et al, 1992), which ranged from 2.1 to 7.3 (mean = 4.1, s.d. = 1.22).

**Immunohistological staining procedures**

**Antibodies sources**

Monoclonal mouse anti-human Bcl-2 oncoprotein (Dako, bcl-2/124, diluted 1:100) recognizes a peptide sequence comprising amino acids 41–54 of Bcl-2 protein (Clearly et al, 1986; Tsujimoto, 1986). The other monoclonal antibodies used were all commercially available and used as described previously (Charpin et al, 1988 a–c; 1993, 1994, 1995a, b; Charpin and Pellissier, 1994): MIB1 and anti-CD-31 (Immunotech, Marseille, France), anti-p53 clone DO-6 (Oncogene Science, Paris, France), anti-cathepsin D (CisBio International, Gif sur Yvette, France), anti-P-glycoprotein (P-gp) clone JSB1-N (Tebu, Le Perray en Yvelines, France), anti-oestrogen and progesterone receptors (ER/PR) (AAbbott kits, Rungis, France).

**Automated immunohistochemistry**

Automated immunohistochemistry (immunoperoxidase) was performed on consecutive sections (4 μm thick) on Ventana 320 device (Grogan et al, 1993; 1995; Galaktionov et al, 1995) (Ventana Medical Systems, Tucson, AZ, USA) with Ventana kits (Ventana Medical Systems, Strasbourg, France) including also aminoethyl carbazol reagent. Sections were counterstained with haematoxylin, dehydrated and mounted in glycergel.

**Image processing and statistical analysis**

Immunostaining was analysed using an Axiophot microscope (Zeiss, Rueil Malmaison, France) and a 3 CCD camera (Sony, Figure 1 Immunohistochemical procedure using frozen sections, anti-Bcl-2 and 124 (Dako, 1:100) avidin–biotin–peroxidase (automated procedure/ Ventana). In invasive ductal carcinoma, the distribution of Bcl-2 protein is heterogeneous, involving not all the tumour cells and partly involving the cytoplasm adjacent to the cell membrane.
and 124 (Dako, 1:100) avidin–biotin–peroxidase (automated procedure/Ventana). High magnification shows that Bcl-2 protein is located in the cell, focally along cell membrane cytoplasm, and along nuclei (invasive lobular carcinoma)

Figure 2 Immunohistochemical procedure using frozen sections, anti-Bcl-2 and 124 (Dako, 1:100) avidin–biotin–peroxidase (automated procedure/Ventana). High magnification shows that Bcl-2 protein is located in the cell, focally along cell membrane cytoplasm, and along nuclei (invasive lobular carcinoma)

Paris, France) and then processed by an image analysis system (SAMBA 2005, Alcatel-TITN, Grenoble, France) (Brugal et al, 1979). The two parameters of densitometric analysis, percentage of immunostained surface (vs counterstained surface) and mean optical density (MOD), which reflects the staining intensity (SAMBA arbitrary units scale ranging from 0 to 255), were obtained as previously reported (Charpin et al, 1988 a–c, 1994, 1995; Charpin and Pellissier, 1994). Statistical analysis was assessed using BMDF (Biomedical Data Package) software (University of California, Berkeley, CA, USA). Various statistical tests were used, depending upon the type (nominal or ordinal) and the distribution (normal or not) of the variables. Consequently, parametric or non-parametric tests were applied, including the chi-square test, Student’s t-test, Kruskal–Wallis test, Mann–Whitney U-test, and the computation of correlation coefficients (Spearman, Kendall and Pearson). In addition, a quantitative index of immunoreactions (QIC) (Maudelonde et al, 1993), combining the percentage of stained surface and mean optical density (MOD), was computed (per cent of stained surface/MOD × 100).

RESULTS

Bcl-2 distribution patterns in cells and tissues

Patterns of immunoreaction are shown and described in detail in Figures 1 and 2. In brief, anti-Bcl-2/124 reacted only with epithelial tumour cells and not with lymphocytes or with stromal cells.

The positive immunoreactions with anti-Bcl-2 did not differ in ductal carcinomas, lobular carcinomas or carcinomas of other types and was observed in cell cytoplasm focally along the cell membrane.

In intraductal carcinomas or in intraductal component of invasive carcinomas the positive Bcl-2 immunostaining exhibited the same pattern as seen in invasive areas. In normal breast present along tumour borders, a positive Bcl-2 reaction was focally observed.

Bcl-2 quantitative immunodetection

Among the 218 tumours probed, 171 (78%) were Bcl-2 positive and 47 were Bcl-2 negative. Distribution of anti-Bcl-2 positive staining evaluated by densitometry on tissue sections is shown on Figure 3A and B. In positive tumours the tumour surface stained by anti-Bcl-2 ranged from 3% to 70% (mean = 22.4%, s.d. = 12.1) (Figure 3A). The Bcl-2 quantitative immunocytochemical index (QIC) varied from 2.6 to 49.2 (mean = 15.2, s.d. = 9.8) (Figure 3B).

Bcl-2 expression and clinicopathological data

Bcl-2-immunostained surface evaluated by image analysis and QIC was independent of the patients’ age, the tumour size, the histological type, lymph node status and the NPI.

In contrast, an inverse relationship was observed between Bcl-2 expression and tumour grades (Table 1), with a significant (P < 0.001) decrease in Bcl-2 expression in high-grade tumours, suggesting that Bcl-2 protein is down-regulated in poorly differentiated tumours.

Bcl-2 expression and quantitative immunodetection of other molecular markers

Results of quantitative evaluation of anti-ER and PR antigenic sites and anti-pS2, of growth fraction (MIB1), and also of
Table 1 Correlation of Bcl-2 expression and tumour grades (218 breast carcinomas)

| Tumour grades | Bcl-2 immunohistochemical expression |
|---------------|-------------------------------------|
|               | Bcl-2 negative (n = 43) | Bcl-2 positive (% stained surface) |
| Grade (Bloom) |                         |                                   |
| 1             | 5 (12%)                 | 27.4% ± 11.5                      |
| 2             | 13 (30%)                | 22.3% ± 9.3                       |
| 3             | 25 (56%)                | 5.6% ± 7.2                        |
| Grade modified (Le Doussal) |           |                                   |
| 1 + 2 (group I) | 8 (18%)                | 24.7% ± 10.5                      |
| 3 (group II) | 10 (23%)                | 20.5% ± 8.4                       |
| 4+5 (group III) | 25 (59%)               | 14.3% ± 5.4                       |

<Chi-square. *Kruskal-Wallis.

Table 2 Correlation of Bcl-2 protein (anti bcl-2/124, Dako) and oestrogen and progesterone receptor antigenic sites (ER, PR) and P-S2 detected by automated (Ventana) and quantitative (SAMBA) immunohistochemistry in 218 breast carcinomas.

| Bcl-2 immunoreaction | Positive surface (%) | OIC* |
|----------------------|----------------------|------|
| ER                   |                      |      |
| Positive             | 24 (±10)             | 16 (±10) |
| Negative             | 14 (±8)              | 11 (±6) |
| ER/PR                |                      |      |
| Positive             | 25 (±13)             | 19 (±12) |
| Negative             | 11 (±9)              | 12 (±10) |
| ER/PR/P-S2           |                      |      |
| Positive             | 24 (±16)             | 12 (±10) |
| Negative             | 11 (±9)              |       |

*Quantitative immunocytochemical index. **Mann-Whitney test.

Table 3 Correlation of Bcl-2 protein expression (anti bcl-2/124, Dako) to cathepsin D CD31, MIB1, P53, P-glycoprotein in 218 breast carcinomas. All antigens were detected by immunohistochemistry on frozen sections, automated procedures (Ventana) and quantitative analysis (SAMBA image analyser)

| Bcl-2 immunoreaction | Bcl-2 positive surface (%) |
|----------------------|----------------------------|
| Positive             |                           |
| Negative             |                           |
| p53                  |                           |
| Positive             | 23/51 (45.1%)             | 28/51 (54.9%) |
| Negative             | 142/166 (85.5%)           | 24/166 (14.4%) |
| P = 0.0001           |                           |
| P-glycoprotein       |                           |
| Positive             | 62/169 (37%)              | 29/47 (62%) |
| Negative             | 107/169 (63%)             | 18/47 (38%) |
| r* = -0.34           |                            |
| Cathepsin D positive |                           |
| CD 31 positive       |                           |
| Positive             |                         | NS |
| Negative             |                         |    |
| Capecitabine positive |                      |       |
| MIB1 positive (Ki67) |                           |
| Positive             |                         | NS |
| Negative             |                         |    |
| r* = -0.30           |                            |
| P = 0.0001           |                            |

*Pearson’s coefficient correlation. **Chi-square.
The prognostic significance of Bcl-2 expression in terms of relapse-free survival and overall survival has been evaluated in some recent studies (Nathan et al, 1994; Silvestrini et al, 1994; Hellemans et al, 1995), using immunohistochemistry of archival fixed tissue samples, and provided discrepant results. Bcl-2 protein expression was not significantly related to a better or worse survival (disease-free survival and overall survival) in the study of Nathan et al (1994). In contrast, in the study by Hellemans et al (1995), although no prognostic value was demonstrated for Bcl-2 protein expression on disease-free survival and overall survival in node-negative breast cancer patients, in node-positive patients Bcl-2 expression was independently related to shortened survival (disease-free and overall survival) using multivariate analysis. In the Silvestrini et al study (1994) Bcl-2 expression in node-negative patients was related to better 6-year survival, but this predictive role of Bcl-2 protein expression was mainly dependent on p53 expression. Thus, it appears that the prognostic significance of Bcl-2 expression in breast carcinomas deserves additional investigations, in larger series, with longer follow-up, and more standardized immunohistochemical assays (work in preparation).

We compared the quantitative evaluation of Bcl-2 expression in tissue with tumour growth fraction as evaluated by MIB1 MAb. Previous reports have shown discrepant results, demonstrating a non-relationship between Ki67-positive immunoreaction and Bcl-2 protein expression (Gee et al, 1994) or an inverse relationship (Johnston et al, 1994). In our series we also observed an inverse relationship between growth fraction and Bcl-2 expression (P < 0.0001).

In contrast, we observed no relationship between Bcl-2 expression and anti-CD-31 (Charpin et al, 1995b) or anti-cathepsin D (Charpin et al, 1993; Maudelonde et al, 1993) positive immunoreactions, suggesting that Bcl-2 involvement in the apoptotic process is independent of angiogenesis and protease synthesis, acting on extracellular matrix digestion. These results also suggest that Bcl-2 is independent of mechanisms of tumour progression.

It has been shown that the bcl-2 gene can inhibit apoptosis triggered by the wild-type p53 gene (Wang et al, 1993). In contrast, the mutant p53 gene can inhibit apoptosis (Lotem, 1993) although it does not seem to have the same large protective range as bcl-2 (Sachs, 1993). An inverse correlation between the expression of Bcl-2 protein and mutant p53 was observed in the MCF 7 breast cancer cell line (Haldar et al, 1994) and in tissue sections of breast carcinomas (Baba et al, 1996; Silvestrini et al, 1994), as in our study, suggesting that mutant p53 could substitute for bcl-2 function in breast cancer cells and that it could also down-regulate Bcl-2 expression (Haldar et al, 1994).

We observed an inverse relationship between Bcl-2 and P-gp expression that is related to multidrug resistance. These results are also paradoxical as Bcl-2 protein has been shown to be involved in chemoresistance (Alnemri et al, 1992; Miyashita, 1992; 1993; Fisher et al, 1993).

Immunostaining of steroid hormone receptors has been shown to be strongly associated with that of Bcl-2 protein (Gee et al, 1994; Johnston et al, 1994; Nathan et al, 1994; Silvestrini et al, 1994; Hellemans et al, 1995; Baba et al, 1996), suggesting that this protein is under oestrogen regulation via oestrogen receptors (Gee et al, 1994). In this respect, the increased expression of Bcl-2 protein in ER-positive tumours of patients treated by tamoxifen shows that Bcl-2 protein expression can be modulated by therapy with anti-oestrogens (Johnston et al, 1994). We observed that Bcl-2 expression was significantly associated with ER-positive
detection in tumours (P ≤ 0.001) and with ER/PR/pS2* (P ≤ 0.001). These results suggest that Bcl-2 protein is probably involved in the response to endocrine therapy. This is unexpected, as mentioned above, as Bcl-2 is thought to prevent programmed cell death and should counteract the tumour-inhibitory effects of endocrine therapy. Indeed, patients whose tumours have strong Bcl-2 immunostaining appear to derive the greatest benefit from endocrine therapy (Gee et al., 1994).

The role of bcl-2 and the significance of Bcl-2 protein in breast carcinomas remains to be elucidated, in particular it remains to be seen whether Bcl-2 expression is involved directly in the pathogenesis of breast carcinomas or, alternatively, whether it is induced secondarily by other genomic changes in tumour cells responsible for modulation of Bcl-2 protein expression. Our results suggest that Bcl-2 protein is related to tumour cell differentiation (grade and hormone receptors), independent of tumour progression (node status, tumour size, stromal angiogenesis and tumour proteasic activity). The potential role of Bcl-2 expression in patient response to endocrine therapy or chemotherapy emphasizes the clinical relevance of Bcl-2 protein immunodetection in tumours. However, the use of Bcl-2 immunohistochemical assays in the monitoring of patient therapy requires optimal technical conditions of detection that can be obtained in automated and quantitative (image analysis) immunohistochemical procedures using frozen tumour samples and well-documented monoclonal antibodies.

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