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c-*erb*B-2 oncoprotein detected by automated quantitative immunocytochemistry in breast carcinomas correlates with patients' overall and disease-free survival

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Summary The prognostic significance of c-*erb*B-2 oncoprotein overexpression detected in tumours by immunocytochemical assays (ICAs) was investigated in 148 breast carcinomas. ICAs were performed under optimal technical conditions with frozen tissue sections and included automated immunoperoxidase technique and computer-assisted analysis (densitometry) of digitized coloured microscopic images. Results of quantitative ICAs (expressed in percentages of c-*erb*B-2-positive surfaces and mean optical densities) were correlated with the patients' follow-up in axillary lymph node-positive (N^+) and node-negative (N^-) subgroups of patients. Patients' follow-up ranged from 9 months (for the first death) to 101 months (for the 121 alive patients) with a 62.5 months mean overall follow-up. It was shown that marked c-*erb*B-2 immunocytochemical expression in tumours (cut-off point 35%) significantly correlated with the patients' poor overall survival in N^+ and in N^- patients (Kaplan–Meier, log-rank test, $P = 0.045$ and $P = 0.015$). Also, marked c-*erb*B-2 immunohistochemical expression correlates with short disease-free ($P = 0.005$), recurrence-free ($P = 0.048$) and metastasis-free survival ($P = 0.05$) (Kaplan–Meier, log-rank test) in N^+, but not in N^- subgroups. It is concluded that in optimal conditions (automated and quantitative ICAs on frozen sections) c-*erb*B immunohistochemical expression is a significant prognostic indicator in terms of overall and disease-free survival. The c-*erb*B-2 protein prognostic significance is independent of node status in terms of overall survival, but not of disease-free survival.

Keywords: automated immunoperoxidase; image analysis; quantitative immunocytochemical assay; c-*erb*B-2; breast carcinomas; patient survival

The prognosis of patients with operable breast cancers is extremely variable. Among the prognostic indices recognized as independent prognostic indicators, axillary nodal status is generally accepted to be the most important prognostic factor in patients with operable cancers. However, axillary lymph node invasion considered as a prognostic factor lacks sensitivity, since 30% of patients with pathologically negative nodes relapse and die within 10 years (McGuire et al, 1992). This observation has led to a search for new accurate prognostic indicators in breast carcinomas.

Molecular biology studies have demonstrated the amplification and overexpression of some oncogenes in breast cancers. Slamon et al (1987, 1989) showed that the c-*erb*B-2 gene was amplified in 27% of breast carcinomas. Further studies confirmed these findings, showing that c-*erb*B-2 amplification could be found in 10–40% of breast cancers (see review in Charpin et al, 1993; Ravdin et al, 1995). Most of these studies revealed that c-*erb*B-2 amplification was correlated with an overexpression (mRNA) and with an increase of the synthesis of p185 kDa-encoded protein. Amplification and overexpression of the c-*erb*B-2 oncogene detected by Southern and Northern blotting have been shown to correlate with the c-*erb*B-2 oncoprotein expression evaluated by Western blotting and immunocytochemistry (Slamon et al, 1987, 1989), suggesting that immunohistochemistry is suitable for evaluating the c-*erb*B-2 gene dysregulation in breast cancers.

The major practical relevance of c-*erb*B-2 amplification and overexpression with increased production of c-*erb*B-2 was first noted by Slamon et al (1987). This report and the following one (Slamon et al, 1989) demonstrated that c-*erb*B-2 was significantly correlated with the clinical outcome of patients with metastatic axillary lymph nodes. Some further publications demonstrated that c-*erb*B-2 amplification and overexpression were significant prognostic indicators, independent of other current prognostic factors (Van de Vijver et al, 1988; Walker et al, 1989; Tandon et al, 1989; Wright et al, 1989; McCann et al, 1991; Paterson et al, 1991; Winstanley et al, 1991). Other studies, however, could not demonstrate that c-*erb*B-2 amplification or overexpression did have prognostic significance (Shou et al, 1989; Heintz et al, 1990; Kury et al, 1990; Clark et al, 1991; McCann et al, 1991; Ravdin et al, 1995). This controversy may arise from variations in the series size, technical procedures and/or statistical methods of analysis (Ravdin et al, 1995; Pauletti et al, 1996). In particular, discrepancies in immunocytochemical studies may result from tissue fixation (Slamon et al, 1987, 1989; Tandon et al, 1989; Clark et al, 1991; Paterson et al, 1991; Winstanley et al, 1991; Piffanelli et al, 1996) and from the lack of standardized means of evaluating immunostaining (Tandon et al, 1989; Charpin et al, 1993).

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In the present study, our goal was to investigate the prognostic significance in terms of overall and disease-free follow-up (8.4 years) of c-*erb*B-2 immunolabelling of 148 breast carcinomas, investigated by immunohistochemistry performed in optimal technical conditions including (1) frozen sections; (2) automated immunohistochemical procedure (Ventana device); and (3) quantification of immunostaining by computer-assisted analysis of digitized microscopic images (SAMBA device).

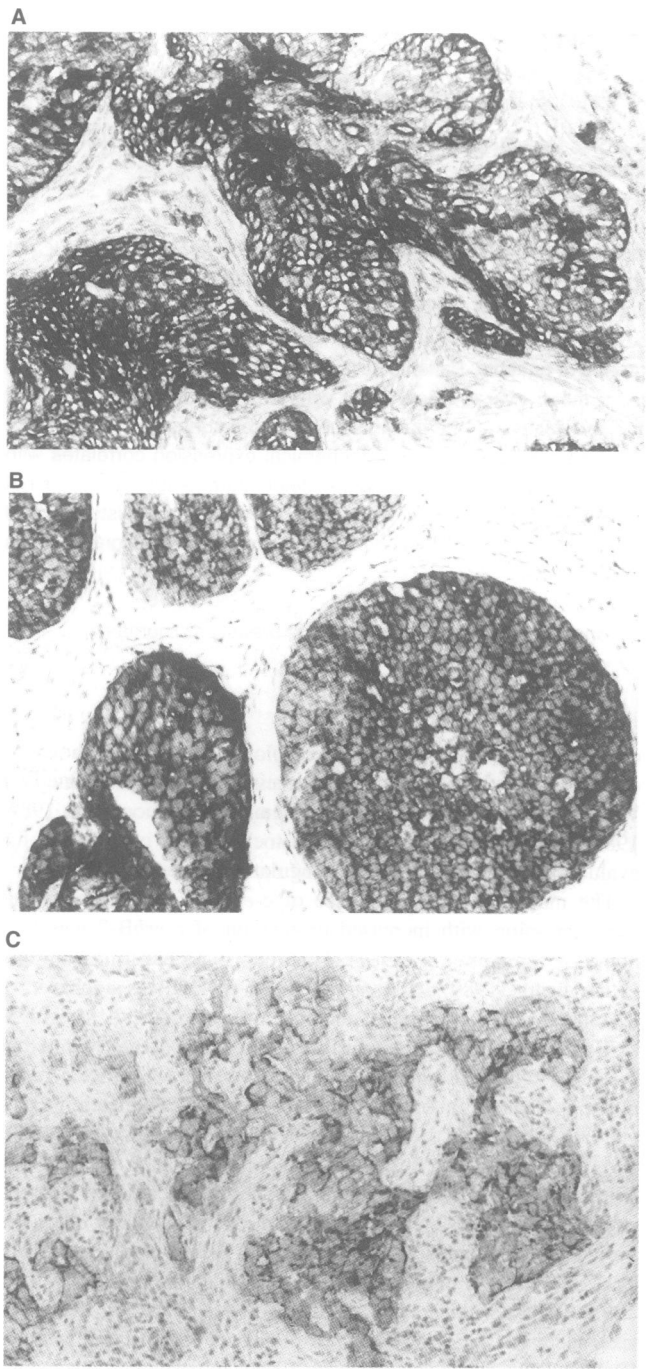

Figure 1 Immunoperoxidase, frozen sections, MAb anti-c-*erb*B-2 protein/CB11 in breast carcinomas. **(A)** Strong positive immunoreaction within cell cytoplasm and cell membrane in ductal invasive carcinoma grade 2. **(B)** Intermediate pattern of staining with heterogeneous distribution of positive reaction in intraductal component of invasive ductal carcinoma. **(C)** Very weak staining in invasive ductal carcinoma grade 2

MATERIALS AND METHODS

Materials

Patients (n = 148) presenting with palpable or impalpable breast carcinomas and who had not received any kind of adjuvant chemotherapy or endocrine therapy were operated on from January 1986 to May 1987. Patients' follow-up ranged from 9 months to 101 months (mean = 62.5 months, s.d. = 25 months; median = 64.9 months). At 101 months (8.4 years), the records showed that 121 out of 148 patients were alive (82%), 27 out of 148 patients (18.2%) were deceased and 20 out of 148 (13.5%) had developed distant metastases, whereas 16 out of 148 (10.6%) had a local relapse. Patients' ages ranged from 34 to 79 years (mean = 53.2 years, s.d. = 12.3 years). The tumours' sizes ranged from 4 mm to 51 mm (mean = 22.5 mm, s.d. = 18.5 mm).

The histological examination of the surgical specimens was performed on paraffin sections stained by haematoxylin, eosin and saffronin. All the tumours were invasive carcinomas. Most (65%) were of ductal type (n = 96/148), whereas the remainder were of lobular type (n = 37/148, 25%) or of various histological types (n = 15/148, 10%), including mucinous (n = 3), tubular (n = 4), medullary (n = 5), papillary (n = 1), apocrine (n = 1) and cribriform mixed (n = 1). Ductal invasive carcinomas (Bloom et al) grade 1 accounted for 16% of the tumours, grade 2 for 59% and grade 3 for 25%. Axillary lymph node dissection was performed in 140 out of 148 patients. The mean number of nodes found in axillary resection was 14.2 (s.d. = 5). The histological examination showed that 66 out of 140 (47%) patients were node positive and 74 out of 140 (53%) node negative. Node-positive patients had more than three metastatic lymph nodes in 21 out of 66 cases.

Immunostaining and image analysis

Tissue

Tissue fragments were sampled for immunostaining by a pathologist immediately after the intraoperative diagnosis performed on frozen sections. The size of the fragments frozen varied according to the tumour size (an average of 5 × 4 × 2 mm). The fragments were sampled in dense tumour areas, lacking grossly visible adipose tissue, promptly dipped in liquid nitrogen and stored at – 80°C in the laboratory tumour library (tumours collected for prospective study since 1986). Immunodetections were performed on 5-µm-thick sections (cryostat Leica 3000, Rueil Malmaison, France).

Immunoperoxidase

Monoclonal (MAb) mouse anti-human c-*erb*B-2 oncoprotein (CB11) was purchased from Biogenex (Menarini, Chevilly Larue, France). Immunoperoxidase technique was performed using an automatic immunostaining device (Ventana, Medical System, Tucson, AR, USA) and Ventana kits (Strasbourg, France) (Grogan et al, 1993, 1995).

Image analysis

Immunostaining was analysed using an Axiophot microscope (Zeiss) and a 3 CCD Sony camera and then processed in an image analysis device (SAMBA 2005, Alcatel TITN, Grenoble, France)

Table 1 Overall survival of patients (8.4-year follow-up) correlated with the c-*erb*B-2 protein immunostained (automated immunocytochemical on frozen sections) surfaces (mean surfaces evaluated by computer-assisted analysis of digitized microscopic images, cut-off point 35%) in 148 invasive breast carcinomas

	c-*erb*B-2 immunostained surface (%)	
Overall survival	**≤ 35%**	**> 35%**
All patients		
Alive	71/121	50/121
Deceased	13/27	14/21
Node-negative patients		
Alive	34/66	32/66
Deceased	7/8	1/8
Node-positive patients		
Alive	34/49	15/49
Deceased	6/17	11/17

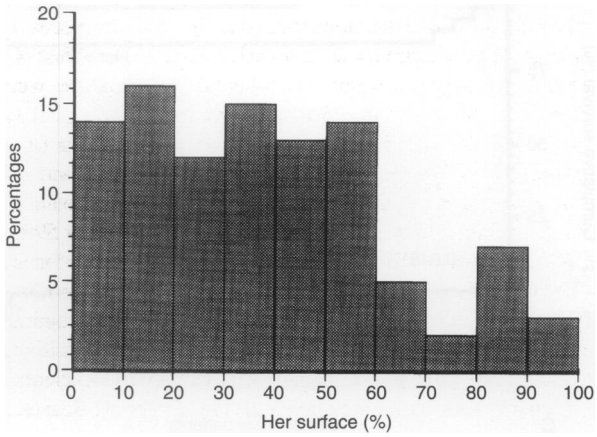

Figure 2 Distribution of c-*erb*B2 (Her-2 neu) immunostained surface in 148 breast carcinomas, evaluated by automated (Ventana) immunoperoxidase on frozen sections: quantitation by computer-assisted analysis (SAMBA) of digitized coloured microscopic images (ordinate, % of patients; abscissa, % of immunostained surface vs counterstained surfaces)

(Brugal et al, 1979). The two parameters of the densitometric analysis, the percentages of positive immunostained surfaces (membrane and cytoplasm) vs counterstained surface and mean optical density (MOD), which depends upon the staining intensity (SAMBA arbitrary units scale: 0–255), were obtained as reported previously (Charpin et al, 1988, 1992, 1993, 1994, 1995*a*, *b*).

Statistical analysis

Analysis of disease-free and overall survival was performed using the Kaplan–Meier method. The difference between curves was evaluated with the Mantel–Cox test (or log-rank test) for censored survival or events observation. All computations were done with BMDP statistical software (University of California, Berkeley, CA, USA). The percentages of c-*erb*B-2 stained surface evaluated by image processing device were stratified and correlated with major events during the course of the disease (distant metastases or local recurrences) and with overall survival in order to define immunohistochemical thresholds of prognostic significance. The optimal c-*erb*B-2 protein cut-off point of positive surface endowed

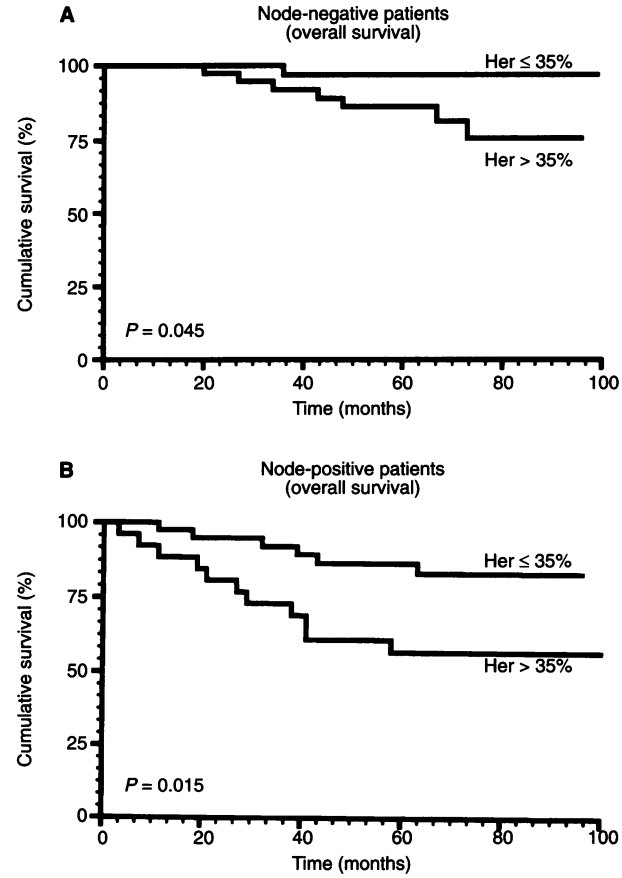

Figure 3 Overall survival (8.4-year follow-up) of patients (n = 148) correlated to c-*erb*B2 (Her-2/neu) protein immunostained surface (mean surface per tumour evaluated by computer-assisted image analysis of digitized microscopic images, cut-off point 35%). **(A)** In node-negative patients and in **(B)** in node-positive patients (Kaplan–Meier, log-rank test, BMDP)

with prognostic significance was determined after statistical validation (Altman et al, 1994).

RESULTS

c-*erb*B-2 distribution in tissue sections

c-*erb*B-2 immunohistochemical expression was similar to that already observed in tumour cells (Charpin et al, 1992, 1993). All the invasive tumours were c-*erb*B-2 positive, but the immunostaining distribution was heterogeneous. Some tumours were markedly positive and others faintly positive (Figure 1). However, the heterogeneity of c-*erb*B-2 expression essentially concerned the surfaces of immunostaining, whereas the intensity of staining was most often homogeneous and strong.

The variation in the c-*erb*B-2-positive surfaces on tissue sections is shown in the histogram in Figure 2. C-*erb*B-2-positive surfaces evaluated by computer-assisted image analysis varied from 0.1% to 98% (mean = 34%, s.d. = 25%). The mean optical densities reflecting the intensity of staining varied from 65 to 91 arbitrary units (mean = 77, s.d. = 13). Since the staining intensity was not significantly variable in the series investigated, this parameter was not used for the correlative studies with the patients' follow-up. For the same reasons, the quantitative immunocytochemical (QIC) index was useless and not worthy of statistical analysis.

Table 2 Disease-free survival of patients (8.4-year follow-up) correlated with c-*erb*B-2 protein immunostained (automated immunocytochemical assays on frozen sections) surface (mean surfaces evaluated by computer-assisted analysis of digitized microscopic images, cut-off 35%) in 148 invasive breast carcinomas

	c-*erb*B-2 protein immunostained surface (%)	
Disease-free survival	**≤ 35%**	**> 35%**
All patients		
No disease	65/108	43/108
Disease	19/40	21/40
Node-negative patients		
No disease	30/58	28/58
Disease	11/16	5/16
Node-positive patients		
No disease	33/46	13/46
Disease	7/20	13/20

Table 3 Recurrence-free survival of patients (8.4-year follow-up) correlated with c-*erb*B-2 protein immunostained (automated immunocytochemical assays in frozen sections) surface (mean surfaces evaluated by computer-assisted analysis of digitized microscopic images, cut-off 35%) in 148 breast carcinomas

	c-*erb*B-2 product immunostained surface (%)	
Recurrence-free survival	**≤ 35%**	**> 35%**
All patients		
No recurrence	78/132	54/135
Recurrence	5/16	10/16
Node-negative patients		
No recurrence	37/65	28/65
Recurrence	4/9	5/9
Node-positive patients		
No recurrence	39/61	22/61
Recurrence	1/5	4/5

Table 4 Metastasis-free survival of patients (8.4-year follow-up) correlated with c-*erb*B-2 immunostained (automated immunocytochemical assays in frozen sections) surface (mean surfaces evaluated by computer-assisted analysis of digitized microscopic images, cut-off 35%) in 148 breast carcinomas

	c-*erb*B2 protein immunostained surface (%)	
Metastasis-free survival	**≤ 35%**	**> 35%**
All patients		
No metastases	75/128	53/128
Metastases	9/20	11/20
Node-negative patients		
No metastases	37/68	31/68
Metastases	4/6	2/6
Node-positive patients		
No metastases	35/53	18/53
Metastases	5/13	8/13

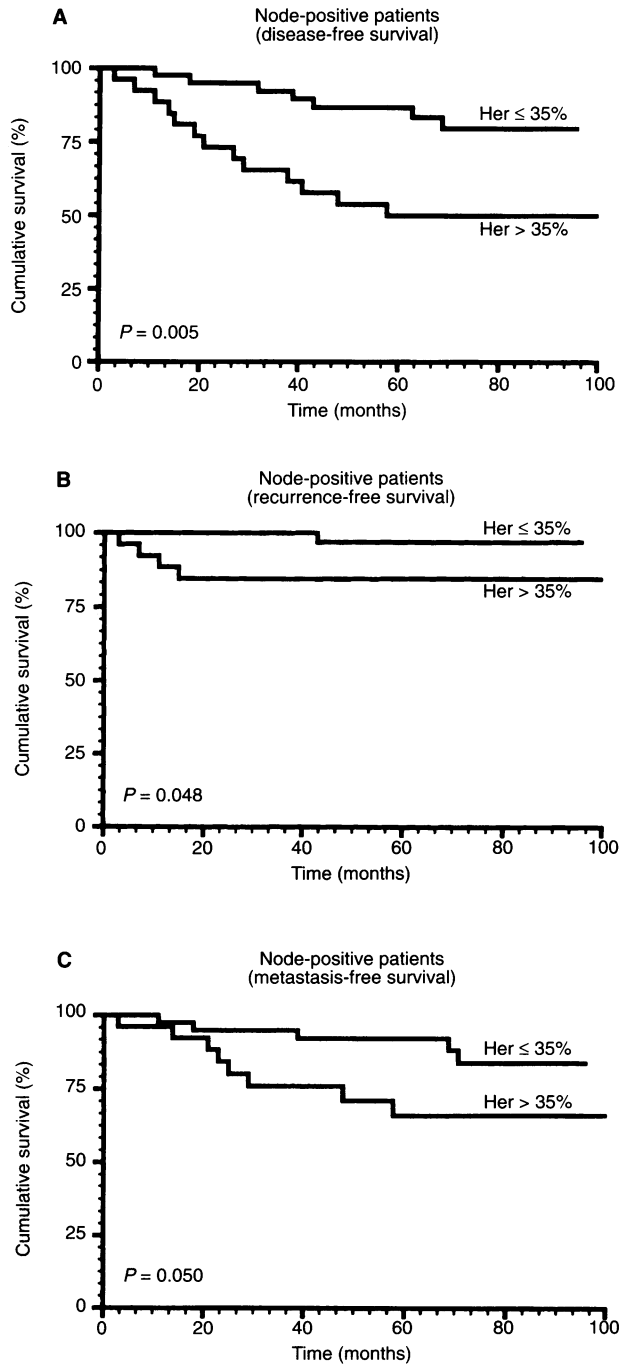

Figure 4 **(A)** Disease-free survival, **(B)** recurrence-free survival and metastasis-free survival (8.4 year follow-up) of patients (n = 148) correlated to c-*erb*B2 (Her-2/neu) protein immunostained surface (mean surface per tumour) evaluated by computer-assisted analysis (SAMBA) of digitized coloured microscopic images, cut-off point 35% in N^+ patients (ordinate, per cent of patients; abscissa, per cent of immunostained surface vs counterstained surface)

c-*erb*B-2 immunohistochemical expression and patients' survival

c-erbB-2 and overall survival (Table 1)

c-*erb*B-2-Immunostained Surfaces (cut-off point 35%) significantly correlated with the patients' overall survival. Tumours with a large c-*erb*B-2-positive surface have a poorer survival than those with a small c-*erb*B-2-positive surface (Table 1). When the entire patient series was stratified into node-positive ($N^+ = 66$) and node-negative ($N^- = 74$) tumours, a significant c-*erb*B-2 (>35%) correlation with poor survival was observed in N^- ($P = 0.045$) patients (Figure 3A) and in N^+ ($P = 0.015$) patients (Figure 3B).

c-erbB-2 and disease-free survival (Table 2)

Marked c-*erb*B-2 protein expression in tissue correlated ($P = 0.015$) with low disease-free survival, but only in N^+ ($P = 0.005$) patients (Figure 4A) not in N^- ($P = 0.17$) patients.

c-erbB-2 protein and recurrence-free survival (Table 3)

Large c-*erb*B-2-positive surfaces also correlated ($P = 0.048$) with low recurrence-free survival in N^+ patients (Figure 4B) but not in the N^- subgroup ($P = 0.23$) of patients.

c-erbB-2 and metastasis-free survival (Table 4)

Marked c-*erb*B-2 protein immunohistochemical expression correlated with metastasis-free survival in N^+ patients ($P = 0.05$) (Figure 4C) but not in N^- patients ($P = 0.1$).

DISCUSSION

The practical clinical relevance of c-*erb*B-2 overexpression is related to its prognostic significance in breast carcinomas (Slamon et al, 1987, 1989) and to its value, recently reported, in predicting response to certain adjuvant therapies (Stal et al, 1995).

The prognostic value of the c-*erb*B-2 oncogene in breast carcinomas has been controversial, although now there is some evidence from the recent literature of its prognostic significance (Borg et al, 1990). Amplification of c-*erb*B-2 has been reported as a significant predictor of both overall survival and relapse time in patients with breast cancers (Slamon et al, 1989; Tandon et al, 1989; Wright et al, 1989) or simply as a predictor of relapse (Van de Vijver et al, 1988; Press et al, 1993).

Furthermore, c-*erb*B-2 amplification has been given a greater prognostic value than most currently used prognostic factors, including hormonal receptors in positive lymph node patients (Slamon et al, 1989) and some of the immunocytochemical studies showed similar results (Gusterson et al, 1988; McCann et al, 1991). Some studies showed that c-*erb*B-2 overexpression was a marker of poor prognosis in N^+ patients (Slamon et al, 1989; Tandon et al, 1989; Borg et al, 1990; Lipponen et al, 1993; Quenel et al, 1995), while others identified c-*erb*B-2 as a marker of poor prognosis in the N^- subgroup of patients (Paterson et al, 1991; Press et al, 1993). In our study, c-*erb*B-2 immunodetectable overexpression correlated with poor overall survival in the N^+ and N^- subgroups of patients, and with shorter disease-free survival only in N^+ patients. However, the lack of statistical prognostic significance in terms of recurrence and metastasis-free survival in N^- patients may result from fewer deaths in the N^- subset compared with the N^+ subset of patients.

Most of the discrepancies in the reports probably result at least partly from the lack of standardized methodologies, particularly for immunohistochemistry.

The amplification and overexpression are correlated with an increase in the encoded protein production detected by Western blots or immunoenzyme assays (Slamon et al, 1989; Quenel et al, 1995; Piffanelli et al, 1996). Clark et al (1991) established that only the expression of five copy amplification could be detected on paraffin sections. The c-*erb*B-2 protein antigenic properties are stable and preserved even after formalin fixation and paraffin embedding. Southern, Northern and Western blots, as well as immunochemistry and fluorescence in situ hybridization (FISH), can be assessed in archival breast cancer specimens to detect c-*erb*B-2 amplification or overexpression, although FISH was recently found to be superior to all methodologies in fixed paraffin-embedded tissue (Pauletti et al, 1996). However, whatever the method used, fixation and paraffin embedding are responsible for a certain loss of antigenicity, as shown by the comparison of immunodetections performed on paraffin sections and on frozen sections (Tandon et al, 1989; Winstanley et al, 1991; Heatley et al, 1993).

Therefore, although paraffin sections are suitable for c-*erb*B-2 immunocytochemical assays (ICAs) c-*erb*B-2 oncoprotein ICAs on frozen sections enable elimination of technical bias as a result of tissue fixation and paraffin embedding. In our view, in order to standardize methodologies, ICAs should be performed on frozen tissue samples, although freezing tissue and storing samples at –80°C is not always easy to achieve.

Similarly, the reproducibility of immunocytochemical tests is better assessed by automated devices, as in our study, than by manual techniques. With the automated devices, many sections can be run at the same time in exactly the same conditions. Tests can be more rapidly assessed and the results provided are more appropriate correlative studies.

Finally, the evaluation of results of the oncoprotein immunodetections by computer-assisted analysis of digitized microscopic images provides for more accurate data than semi-quantitative analysis, which depends on observer experience and subjectivity. Moreover, results of densitometry by image analysis of tissue sections consist in quantitative data also more appropriate for statistical analysis, in particular for determining the cut-off point of prognostic significance.

Quantitative immunocytochemistry has already been developed on a different system for c-*erb*B-2 ICAs (Baak et al, 1991; Bacus et al, 1990*a*, *b*; Charpin et al, 1992, 1993; Press et al, 1993). In one study (Press et al, 1993), the quantitative c-*erb*B-2 ICAs were related to the patients' follow-up. In this study, detection was performed on paraffin sections using a polyclonal antibody. It was shown that breast carcinomas with high overexpression of c-*erb*B-2 oncoprotein were associated in node-negative patients with a risk of recurrence 9.5 times greater than those with low c-*erb*B-2 expression (Press et al, 1993). In our study, we obtained different results, although we also used computerized image analysis, probably because we used different antibodies and frozen-tissue samples. Quantitative immunochemistry, as assessed in our study, was developed to standardize immunohistochemical assays. The term, quantitative assay, only refers to quantitation of the immunostaining and cannot, therefore, pretend to quantify the antigens themselves, since immunohistochemical signals are the results of a series of amplification reactions that may not reflect the true receptor level within the samples.

In conclusion, using automated (Ventana device) and quantitative (SAMBA system) immunohistochemical assays on frozen-tissue samples of breast carcinomas, we showed that strong c-*erb*B-2 expression (cut-off point 35%) correlated with poorer 8-year overall survival in N^+ and N^- patients and shorter disease-free survival in N^+ patients compared with weak c-*erb*B-2 immunoexpression.

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