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Acknowledgements—We are grateful to Professor Marc Feldmann for providing the antiserum, Mrs Beverley Richardson and Mrs Wendy Pitts for typing the manuscript.

Eur J Cancer, Vol. 28, No. 2/3, pp. 644–648, 1992.
Printed in Great Britain

0964–1947/92 $5.00 + 0.00

Overexpression of the c-*erb*B-2 Oncoprotein: Why does this Occur More Frequently in Ductal Carcinoma *in situ* than in Invasive Mammary Carcinoma and is this of Prognostic Significance?

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Overexpression of c-*erb*B-2 occurs in 60% of *in situ* and 25% of infiltrating ductal carcinomas. We have previously found very strong associations between immunohistochemical staining for c-*erb*B-2 and histological pattern and nuclear size in ductal carcinoma *in situ* (DCIS) and less strong correlation with proliferative activity. In a further study of infiltrating ductal carcinomas we have found that, in addition to tumours arising from c-*erb*B-2 positive, large celled, rapidly proliferating, comedo carcinomas and c-*erb*B-2 negative small celled cribriform/micropapillary carcinomas with a low proliferative rate, there is a third group of c-*erb*B-2 negative tumours with large nuclei and variable proliferative activity. These latter tumours are not seen in pure DCIS suggesting that they have a very transient *in situ* stage. Therefore, although in pure DCIS c-*erb*B-2 positivity appears to be associated with tumours with a greater invasive potential, and c-*erb*B-2 negativity with tumours having a more favourable prognosis, the latter is not necessarily true in infiltrating disease.
Eur J Cancer, Vol. 28, No. 2/3, pp. 644–648, 1992.

INTRODUCTION

AMPLIFICATION OF the c-*erb*B-2 oncogene and overexpression of the oncoprotein have been associated with poor prognosis in patients with infiltrating breast carcinoma [1]. Early papers reported a 35% difference in survival at 4 years for node positive patients with c-*erb*B-2 positive tumours [1]. This finding was emphasised in later studies with large numbers of patients [2–5]. Other smaller studies on fewer than 200 patients failed to show any significant difference, suggesting that any prognostic significance was weak [6]. Although the majority of studies which have included a large number of patients do find an association between c-*erb*B-2 oncogene enhancement and poor prognosis, a recent study by Clark and McGuire [7], of 362 patients failed to do so.

Other inconsistencies in the relationship between c-*erb*B-2 overexpression and mammary carcinoma are related to its correlation with tumour type. Whilst in studies of infiltrating carcinoma the proportion of tumours showing overexpression has ranged from 10 to 30% [8, 1] in carcinoma *in situ* the incidence of overexpression is much higher, in the region of 60% [9–11]. We have undertaken several studies of c-*erb*B-2 overexpression in mammary ductal carcinoma and have been particularly impressed by this latter finding [12, 13]. We, therefore, undertook the present study in order to investigate this further by reviewing our previous work, which concentrated on *in situ* tumours (studies 1 and 2), and by carrying out a new study of the detailed morphology of infiltrating ductal carcinomas (study 3).

MATERIALS AND METHODS

c-erbB-2 staining

All studies concentrated on ductal carcinomas. In all cases the histological pattern of the *in situ* component was evaluated; the nuclear size of the cells was measured and, in two of the studies, cellular proliferation was also measured. All studies were carried out on formalin fixed paraffin embedded sections using polyclonal antibody 21N [14], as previously described [15]. Sections were floated onto poly-L-lysine coated slides and allowed to dry overnight at room temperature without heat. A peroxidase conjugated avidin–biotin technique was used to demonstrate the antibody/antigen reaction. Inter- and intra-assay reliability and consistency was maintained by including positive and negative controls for each batch of staining. Staining was repeated for any batch in which the controls were unsatisfactory. Only membrane staining was considered to be indicative of the presence of the c-*erb*B-2 protein. Cytoplasmic staining, in the absence of membrane staining, was disregarded. The presence of any membrane staining was taken as positive. Intensity and number of cells staining was not used in this analysis, although there was a tendency, that as the proportion of cells showing a positive reaction increased so did the intensity.

Measurement of nuclear size

The size of the nuclei was measured with a graticule in a 10 × eyepiece calibrated with a stage micrometer, as previously reported for pure ductal carcinoma *in situ* (DCIS) [12]. Measurement of nuclear size was preferred to the measurement of cell size as, in many cases, cell margins were indistinct. In our previous studies of DCIS [12, 13] tumours were assigned to one of four categories according to the size of the nuclei: 1-small, 2-intermediate, 3-large, and 4-mixed. In this present study tumours have been divided into two groups; 1-small, containing only nuclei measuring up to 10 μm, and 2-large, containing any nuclei measuring more than 10 μm, although the majority of the latter group contained at least some nuclei measuring more than 15 μm. Thus the previous categories 2, 3 and 4 have been combined.

Proliferative activity

Thymidine labelling. Labelling of the tissue with thymidine and determination of the index were carried out as previously described by Meyer and Connor [16].Slices of tissue were incubated with tritiated thymidine for 2 h within 2 h of excision of the tumour. For determination of the index 2000 neoplastic cells were counted. Cells counted as being labelled had at least five times more grains of silver deposited over the nuclei than had the background. Tumours were categorised into two groups based on the labelling index of the intraductal component. A previously determined cut-off of 2% was used to categorise tumours into those with a low or high thymidine labelling index (TLI) [17].

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Received and accepted 24 Oct. 1991.

Table 1. Comparison between nuclear sizes and c-erbB-2 immunoreactivity in the three studies of ductal carcinoma of the breast

	Study 1[12]*	Study 2[13]†	Study 3‡
Small nuclei	23	21	30
c-*erb*B-2 + ve (%)	0	1 (4)	2 (7)
Large nuclei	49	40	118
c-*erb*B-2 + ve(%)	44 (90)	18 (45)	37 (31)
Total number	72	61	148
c-*erb*B-2 + ve (%)	44 (61)	19 (31)	39 (26)

* Pure DCIS.
† *In situ* component of tumours with a small, variable infiltrating component.
‡ Infiltrating ductal carcinoma.

DNA flow cytometry. Flow cytometric DNA analysis was performed on cell suspensions prepared from 50 μm paraffin-embedded, formalin-fixed sections using the method of Camplejohn [18] as described by O'Reilly *et al* [19]. Tumours were classified into two groups, those with a high S-phase fraction (SPF) and those with a low SPF according to whether the percentage of cells in S-phase was above or below the median value of 8% taken from previous studies of a wide range of mammary carcinomas [20].

Statistical analysis

The significance of differences in proportions between different groups was evaluated using the χ^2 test. The magnitude and significance of correlations between variables was measured by Pearson's correlation coefficient.

RESULTS

Study 1—pure mammary carcinoma in situ *(DCIS)* [12]

In our previous immunohistochemical study of 72 cases of pure DCIS c-*erb*B-2 immunoreactivity was seen in 61% of tumours [12]. There was a significant correlation between histological pattern and c-*erb*B-2 overexpression ($\chi^2 = 44.2$, $P < 0.0001$) and an even closer correlation between nuclear size and c-*erb*B-2 positivity ($\chi^2 = 54.3$, $P < 0.0001$). In this study of pure DCIS 68% of the tumours contained cells with large nuclei and 32% of tumours were composed only of cells with small nuclei (Table 1). Large cell comedo carcinomas were nearly always positive and small cell cribriform/micropapillary carcinomas were never positive. The majority of mixed histological patterns contained some cells with large nuclei and these cells were also c-*erb*B-2 positive [12].

*Study 2—*in situ *component of ductal carcinomas with a variable infiltrating component* [13]

In our second study 61 ductal tumours of this type were studied, these tumours formed part of a larger previous study [13] in which all the carcinomas had a large *in situ* component, although a varying amount of infiltrating tumour was also present in the majority. c-*erb*B-2 immunoreactivity was seen in the *in situ* component in 19 (31%) of these tumours. In this study the proportion of tumours containing cells with large nuclei was 66%, those with only small sized nuclei was 34% (Table 1). Although the correlations between c-*erb*B-2 immunoreactivity, pattern of DCIS and size of the nuclei was high, the significance was less than that seen in the study of pure DCIS

Table 2. Features studied in 148 *infiltrating carcinomas in study* 3

	No. of cases		*n* (%)
c-*erb*B-2 staining	148	Negative	109 (74)
		Positive	39 (26)
Nuclear size	148	Small	30 (20)
		Large	118 (80)
S-phase fraction	127	< 8%	61 (48)
		> 8%	66 (52)

*Table 3. Relationship between histological pattern and c-*erb*B-2 immunoreactivity in the in situ component of the infiltrating carcinomas in study 3*

	n	c-*erb*B-2 +ve (%)	
Cribriform/ micropapillary	30	2 (7)	
Mixed	74	21 (28)	χ^2 18.05
Comedo/solid	11	8 (73)	$P < 0.0001$
Total	115	31 (26)	

(histological pattern; $\chi^2 = 28.8$, $P < 0.0001$, nuclear size; $\chi^2 = 19.7$, $P < 0.0001$). The most striking difference was seen in the tumours of mixed histological pattern. In the study on pure DCIS the majority of these (15/18) were c-*erb*B-2 positive [12], whereas in the second study only 4/18 were c-*erb*B-2 positive [13]. This difference is statistically significant (χ^2 11.15, $P < 0.001$). In this second study an additional parameter of proliferative activity of the *in situ* component measured by thymidine labelling index was included and a significant association was found between the presence of c-*erb*B-2 protein and high proliferative activity ($P = 0.02$) [13].

Study 3—infiltrating ductal carcinoma with a minimal or absent in situ *component*

The basis of this present, new study was formed by 148 primary infiltrating ductal breast carcinomas and were part of a larger study investigating the relationship between c-*erb*B-2 oncoprotein expression and SPF with prognosis [20]. The patients all had primary, operable breast cancer diagnosed at Guy's Hospital between 1980 and 1983 and were selected from the original 172 cases on the basis of histological subtype. The distribution of the various factors studied in these 148 carcinomas is shown in Table 2. c-*erb*B-2 immunoreactivity was seen in 39 (26%) of the tumours.

Comparison of features within in situ *and infiltrating components.* In 33 of the 148 cases no *in situ* component was present in the sections which were stained for c-*erb*B-2 protein, although areas of *in situ* disease were present in other sections in 12 of these 33 cases. Comparisons were made of both the presence of c-*erb*B-2 staining and the nuclear size of the malignant cells between the *in situ* and infiltrating components of the 115 tumours, where both were present. c-*erb*B-2 staining was identical in 113/115 tumours (98%). There was no staining in 82 and positive staining in 31. In the two discrepant cases the *in situ* component did not stain, but the infiltrating component was weakly positive.

The nuclear size was identical in 103/115 cases (90%). In two cases the *in situ* component contained larger nuclei than the infiltrating component, and in 10 cases the reverse was true.

In situ *pattern.* The histological patterns of the *in situ* component in the 115 infiltrating carcinomas is shown in Table 3. Two thirds (64%) of tumours had a mixed pattern, 20% had a cribriform/micropapillary pattern and only 10% of the tumours were of the comedo/solid pattern. The proportion of tumours with *in situ* components of mixed pattern is considerably higher in this group of infiltrating tumours than in either of our previous studies (cf. study 1, 29%; study 2, 20%; study 3, 64%). The incidence of c-*erb*B-2 immunostaining is also shown in Table 3. As has been shown in pure and predominantly *in situ* carcinomas (studies 1 and 2) cribriform/micropapillary tumours rarely stained (2/30), whilst positive staining was seen in most (8/11) of the tumours with a comedo/solid pattern. In contrast to the study on pure DCIS where the majority of tumours with a mixed histological pattern stained, only 28% of them stained in this study.

*Evaluation of c-*erb*B-2 staining, nuclear size and S-phase fraction.* Relationships between the presence of staining for c-*erb*B-2 protein and the size of the nuclei found in the 148 infiltrating carcinomas is shown in Table 1. In this study 80% of the tumours contained cells with large nuclei and 20% were composed only of cells with small nuclei. The association between nuclear size and c-*erb*B-2 immunoreactivity was much weaker than in the previous two studies ($P < 0.02$), but still significant. Staining was seen in only 2 of the 30 tumours composed of cells with small nuclei. Approximately one third (37/118) of the tumours containing large nuclei stained positively. Thus, although c-*erb*B-2 staining is nearly always confined to tumours which contain large nuclei, not all of the infiltrating ductal carcinomas which contain large nuclei are c-*erb*B-2 positive. c-*erb*B-2 protein is hardly ever expressed in tumours composed of small nuclei. Table 1 also shows that as the proportion of the infiltrating component increases from zero, in study 1, to a major contribution, in study 3, the percentage of tumours with cells with large nuclei expressing c-*erb*B-2 protein decreases from 90% in study 1 to 45% in study 2, and 31% in study 3.

As the quality of flow cytometric data was maintained by rejecting samples with a coefficient of variation greater than 8% and as, additionally, it is not possible to calculate SPF for multiploid tumours, the number of cases with valid SPF data was reduced from 148 to 127. A significant association was found between c-*erb*B-2 positivity and high SPF ($\chi^2 = 4.97$, $P < 0.03$). Of the 127 tumours 66 had a high SPF and 22 of these were c-*erb*B-2 positive, it follows that there were 44 tumours with a high SPF which were c-*erb*B-2 negative. There were also a few tumours with a low SPF which were c-*erb*B-2 positive (see Table 4). The proliferation rate in this group of infiltrating tumours was significantly associated with nuclear size ($\chi^2 = 10.93$, $P < 0.005$) but this was not quite as strong as the association seen between size and TLI in study 2 ($\chi^2 = 24.42$, $P < 0.0001$).

The relationships between c-*erb*B-2 expression, SPF and nuclear size was examined in the 127 tumours with adequate SPF data and is shown in Table 5. SPF was available for 24 of the tumours containing cells with small nuclei only one of which

Table 4. Relationship between c-erbB-2 immunoreactivity and SPF in study 3

	SPF		
	> 8%	< 8%	
c-*erb*B-2 +ve	22	9	31
−ve	44	52	96
Total	66	61	127

$\chi^2 = 4.97, P < 0.03$.

Table 5. Relationship between nuclear size and rate of proliferation in study 2 and study 3

Study 2	*n*	c-*erb*B-2 +ve *n* (%)	High TLI *n* (%)
Small	18	1 (5)	3 (17)
Large	43	19 (44)	31 (72)
Total	61	19 (31)	34 (55)
Study 3	*n*	c-*erb*B-2 +ve *n* (%)	High SPF *n* (%)
Small	24	1 (4)	11 (45)
Large	103	30 (29)	55 (54)
Total	127	31 (24)	66 (52)

expressed the oncoprotein whilst 11 (45%) had a high SPF. For the 103 tumours containing cells with large sized nuclei, 30 (29%) were c-*erb*B-2 positive and 66 (52%) had a high SPF. Thus a higher proportion of tumours had a high rate of proliferation than expressed c-*erb*B-2 for both nuclear sizes. A summary of the similar TLI data from study 2 is also shown in Table 5.

DISCUSSION

The aim of this work was to investigate the reasons for the difference in the incidence of c-*erb*B-2 immunoreactivity between pure *in situ* and infiltrating ductal carcinomas. In study 3 the degree of c-*erb*B-2 staining was very similar in the *in situ* and infiltrating components. This confirms our previous observation [21], and those of Borg *et al.* [22] and Iglehart *et al.* [23] and shows quite clearly that there is no loss of c-*erb*B-2 protein as tumours progress to invasion from a pure *in situ* state. Therefore, there must be some other reason why fewer infiltrating tumours overexpress the protein. The nuclear sizes of the *in situ* and infiltrating components were also very similar and, as we have found previously for *in situ* disease [12, 13] almost all of the c-*erb*B-2 positive cases contained some large nuclei.

Our studies of pure *in situ* carcinoma have clearly shown that the tumours containing large nuclei are nearly always c-*erb*B-2 positive. This does not appear to be true for infiltrating tumours where, although the majority of c-*erb*B-2 positive tumours contain cells with large nuclei, a significant proportion of tumours with large nuclei are c-*erb*B-2 negative. As the cell size of the *in situ* and infiltrating components in study 3 was very similar, a change in cell size cannot explain this difference.

There is also a relationship between proliferative activity and c-*erb*B-2 positivity, although this is not strong. In both our studies in which proliferative activity was assessed the c-*erb*B-2 positive cases tended to be rapidly proliferating (study 2, 75%; study 3, 71%, Table 4). There were, however, a number of tumours in both studies with high rates of proliferation not accompanied by c-*erb*B-2 positivity. Indeed in study 3 two thirds of the tumours had a high rate of proliferation but did not express c-*erb*B-2 (Table 4).

Three other studies have examined the relationship between overexpression of c-*erb*B-2 and SPF in infiltrating carcinoma. Anbazhagan and colleagues [24] found a significant correlation between staining and SPF in a univariate analysis on 110 patients ($P = 0.02$). This significance was lost in a multivariate analysis. Baak *et al.* [25] also found a weak but significant ($P = 0.04$) association between overexpression of c-*erb*B-2 and SPF. In contrast, Borg *et al.* [26] found a very strong relationship between amplification of the oncogene and SPF in 487 patients ($P < 0.00001$); as in our series the majority of the tumours with c-*erb*B-2 amplification (80%) had a high SPF, but these tumours only made up one quarter of the cases with a high rate of proliferation. Two studies which failed to find an association between c-*erb*B-2 positivity and proliferation have been reported by Kommoss *et al.* [27] and Bacus *et al.* [28]. In both studies Ki67 immunoreactivity was used as the index of proliferation and there is still some uncertainty as to what Ki67 measures. In summary it would appear that whilst there is an association between c-*erb*B-2 overexpression and proliferative activity, this is weak.

Having established relationships between c-*erb*B-2 overexpression and nuclear size, and c-*erb*B-2 overexpression and proliferation, we looked at the inter-relationship between these three factors. In our study of the *in situ* component of tumours with a variable infiltrating component (study 2) tumours with small nuclei were c-*erb*B-2 negative and had a low TLI. The majority of tumours which expressed c-*erb*B-2 had large nuclei and a high TLI. However, there was a small proportion of tumours with large nuclei and a high TLI which were c-*erb*B-2 negative (Table 5). The inter-relationships were much less clear cut in study 3. Although, as in study 2, almost all of the tumours with small nuclei were c-*erb*B-2 negative a number of these tumours had a high SPF. Whilst the majority of c-*erb*B-2 positive tumours had large nuclei and a high SPF there were many tumours with large nuclei which were c-*erb*B-2 negative, over half of which had a high rate of proliferation.

These data suggest that there are at least three groups of infiltrating tumours. (1) Those composed of cells with small nuclei, which have arisen from small celled cribriform/micropapillary DCIS. These have a low rate of proliferation and of c-*erb*B-2 overexpression. (2) Tumours composed of large cells which have arisen from large celled comedo DCIS. These have a high rate of proliferation and of c-*erb*B-2 overexpression. (3) Tumours composed of cells with variable nuclear sizes, but including some large nuclei, over half of which have a high rate of proliferation but none of which overexpress c-*erb*B-2.

Our hypothesis is that the latter group of tumours only have a transient *in situ* period and quickly become invasive. Because of this rapid progression to invasion, these tumours were not found in our first study of pure DCIS, they made only a minor contribution to our second study of tumours with a prominent DCIS component accompanied by a variable infiltrating component but have become very obvious in this third study of infiltrating tumours. This could explain the "dilution" of overall

c-*erb*B-2 positivity seen in studies of infiltrating tumours when compared to pure *in situ* tumours.

If this is so, it could be accepted that the presence of c-*erb*B-2 overexpression is a marker of poor prognosis, since the c-*erb*B-2 positive *in situ* tumours are always composed of large cells, usually of comedo pattern and there are data to suggest that such tumours have a greater invasive potential than other patterns of *in situ* carcinoma [17, 29, 30]. In cases of infiltrating carcinoma the c-*erb*B-2 positive tumours again contain large cells and are rapidly proliferating, both factors being associated with a poor prognosis.

In contrast, lack of c-*erb*B-2 overexpression, is not necessarily reassuring. In the case of *in situ* carcinoma it may well be a good prognostic sign as it occurs in tumours composed of small cells usually of cribriform/micropapillary pattern and there are data to suggest that these tumours are less likely to become invasive than are those of comedo pattern [17, 30, 31]. On the other hand, in the case of infiltrating carcinoma, lack of c-*erb*B-2 expression may not be such good news. Whilst tumours with small nuclei and tumours with low proliferative activity are nearly always c-*erb*B-2 negative, there are also significant numbers of c-*erb*B-2 negative tumours which contain at least some large cells, and many of these tumours have a high rate of proliferation. As already suggested it is possible that this group of tumours has only a transient *in situ* stage.

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Acknowledgements—The authors are extremely grateful to Dr Michael Richards, of Guy's Hospital and Dr John Meyer, of St Louis, Missouri, USA, for helpful discussions and valuable criticism during the preparation of this manuscript.