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# Proliferating cell nuclear antigen in breast lesions: correlation of c-*erb*B-2 oncoprotein and EGF receptor and its clinicopathological significance in breast cancer

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**Summary.** Monoclonal anti-proliferating cell nuclear antigen (PCNA PC10), which is directed against a 36 kDa auxiliary protein for DNA polymerase delta specific for the S-phase of cell cycle, was used to measure tumour cell proliferation in 4 lactating breasts and 98 benign and malignant breast tumours. The percentage of PCNA-positive cells determined by point counting was significantly lower in the lactating breast [mean 3.6%, standard deviation (SD) 0.67, $n=5$] than in fibroadenoma and mastopathy (mean 23.7, SD 5.0, $n=2$). Primary breast carcinoma showed a PCNA index ranging from 2% to 36% (mean 12.3, SD 9.3, $n=50$), whereas in recurrent carcinoma the index was mean 28.5, SD 4.0. A high index was correlated with c-*erb*B-2 and epidermal growth factor (EGF) receptor membrane reactivity, worsening histological grade, poor survival and disease-free survival. The expression of c-*erb*B-2 and EGF receptor was associated with poor survival and disease-free survival in primary breast cancer patients.

**Key words:** Proliferating cell nuclear antigen – c-*erb*B-2 – Epidermal growth factor receptor – Breast lesions

## Introduction

Proliferating cell nuclear antigen (PCNA) is a 36 kDa auxiliary protein for DNA polymerase delta, the expression of which is correlated with the S-phase of the cell cycle (Bravo and Celis 1980; Celis and Celis 1985; Hall et al. 1990; Miyachi et al. 1978; Scott et al. 1991; van Dierendonck et al. 1991). Cell kinetics is an important adjutant to histologically based tumour classification and an important indicator of treatment response and relapse in many types of cancer (Tubiana et al. 1989).

Tumour cell kinetics in the prediction of disease-free survival and short-term survival in breast cancer patients have been carried out on the basis of thymidine incorporation and flow cytometry, and alternative methods of assessment with Ki-67 antibodies that recognize antigens associated with cell proliferation (Dervan et al. 1989; Isola et al. 1990; Lele et al. 1987; McGuire 1987; Silvestrini et al. 1986; Tubiana et al. 1989). Cell-cycle-related activities using PCNA, bromodeoxyuridine (BrdU) and Ki-67 staining have been reported in cultured breast and ovarian cancer (van Dierendonck et al. 1991). Argyrophilic nuclear organizer regions (AgNORs) in breast carcinomas were correlated with Ki-67 scores (Dervan et al. 1989). Flow cytometric assessment of cell proliferation in breast cancer was recently shown to correlate with PCNA immunoreactivity (Garcia et al. 1989) and with AgNORs (Giri et al. 1989).

The c-*erb*B-2 oncogene is amplified and overexpressed in a number of human adenocarcinomas (McCann et al. 1990). Recent studies have indicated the association of c-*erb*B-2 amplification with poor survival and early recurrence in breast cancer patients and is assumed to play an important role in malignant transformation (Paik et al. 1990; Slamon et al. 1989; Tandon et al. 1989; Tsuda et al. 1989; Walker et al. 1989; Wright et al. 1989). The epidermal growth factor (EGF) receptor is homologous with c-*erb*B-1 and is closely related, but distinct from c-*erb*B-2 oncogene (Coussens et al. 1985; Semba et al. 1985; Tsutumi et al. 1990). The activation of EGF receptor by EGF or transforming growth factor (TGF-alpha) induces a mitogenic signal in the cell (Hunts et al. 1985; Ullrich et al. 1984; Yamamoto et al. 1986). Expression of EGF receptor protein in established breast tumours has been reported to be an index of poor prognosis and to predict the likelihood of lymph node involvement and lack of response to endocrine therapy (Battaglia et al. 1988; Nicholson et al. 1989; Sainsbury et al. 1987).

This study describes the proliferating index of cancer cells on the basis of PCNA immunohistochemistry. We set out to compare the status of c-*erb*B-2 and EGF receptor in the tumours with tumour size, lymph node status, clinical stage and histological grade, and correlated these with the clinical outcome.

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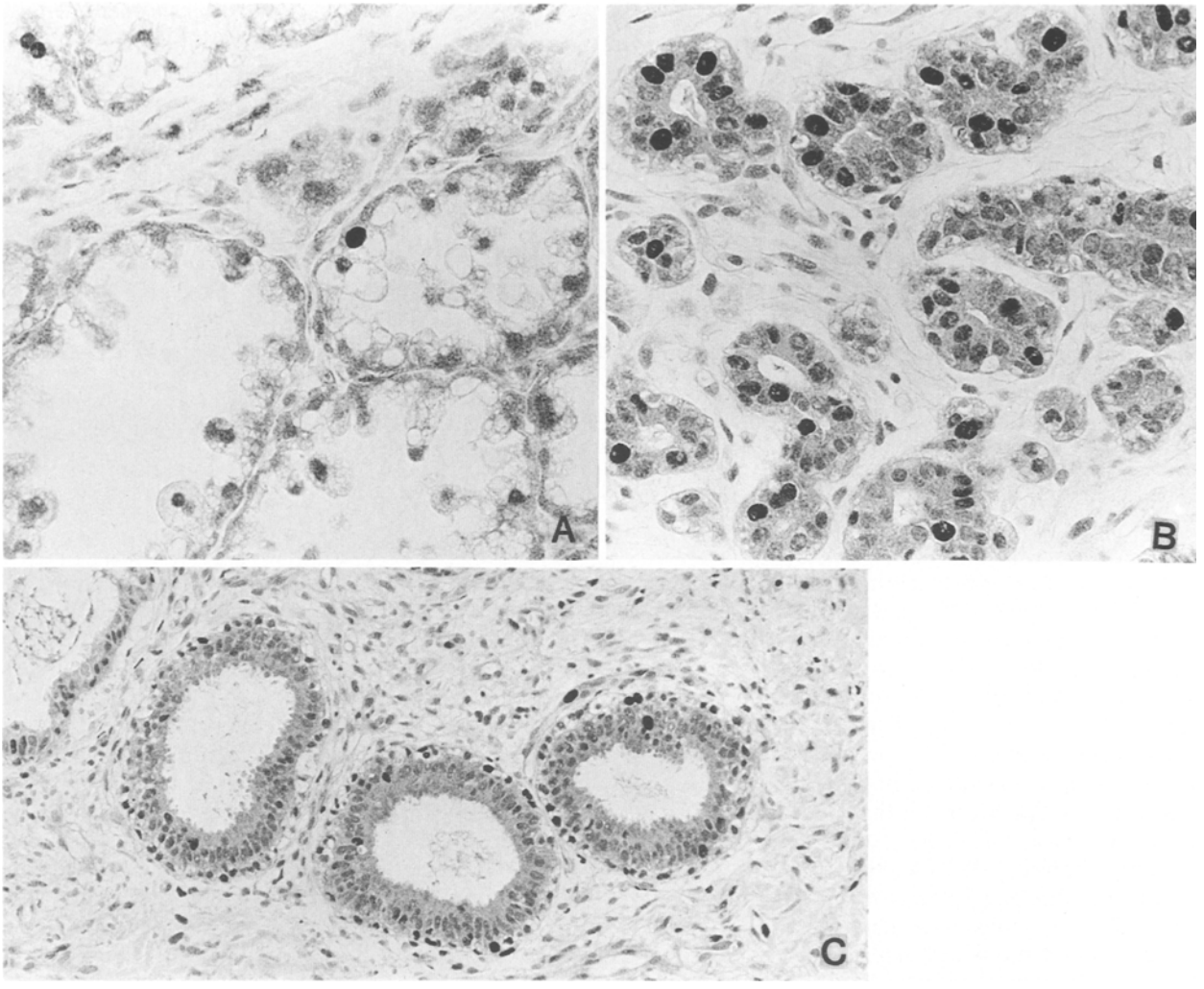


**Fig. 1A–C.** Proliferating cell nuclear antigen (PCNA) immunostaining in the breast lesions. The sections are stained with PCNA, with counter staining by methyl green. **A** Lactating gland. A few PCNA-positive cells (5.5%) are scattered in lactating mammary epithelium. **B** Mastopathy. Frequency of PCNA-positive cells is 19.8%; and positive cells are located in tubular structures. **C** Mastopathy. Frequency of PCNA-positive cells is 30.7% and dilated duct-like structure contains positive PCNA cells in both outer and luminal layers. **A, B** ×200; **C** ×100

## Patients and methods

A total of 50 patients presenting with primary breast cancers who had undergone radical or modified radical mastectomy at the Department of Surgery, Kinki University School of Medicine between January 1981 and July 1986 were evaluated. Benign breast lesions (12 fibroadenomas, 14 cases of mastopathy and 5 normal lactating breasts) were also evaluated for comparative study. The clinical stage of the tumour at the time of surgery was defined according to the TNM system. The histological classification of breast carcinoma used was that of the Japanese Breast Carcinoma Society (1989). Carcinoma was classified into non-invasive carcinoma and invasive which subdivided into invasive ductal carcinoma, papillotubular carcinoma, solid-tubular carcinoma (medullary tubular carcinoma) and scirrhous carcinoma. Special types of carcinomas (mucinous medullary, invasive lobular, adenoid cystic, squamous cell, spindle cell, apocrine tubular, secretory and others were noted).

The tumour tissue was routinely processed, fixed in formalin and embedded in paraffin for routine histopathological examination and serial sections at 4 μm from the blocks were used for immunohistochemical study of PCNA, c-*erb*B-2 oncoprotein and EGF receptor expression by using a three-stage streptavidin-biotin immunoperoxidase method. Monoclonal anti-PCNA (Dakopatts, Denmark) and monoclonal anti-EGF receptor (Biomarker, Israel) was used at a dilution of 1:20 and 1:80 respectively for 1 h. Anti c-*erb*B-2 polyclonal antibody was obtained from Nichirei (Japan) and the sections were incubated overnight at 4° C at a dilution of 1:50. Endogenous peroxidase activity was blocked by methanol-contained 0.05% $H_2O_2$ for 30 min, and sections were treated with normal rabbit serum (1:20) for 30 min for non-specific background staining. They were then incubated in antibodies and visualized by diaminobenzidine (DAB) containing 0.05% $H_2O_2$ solution. All other secondary reagents were purchased from Dakopatts. The sections with PCNA staining were counterstained with methyl green.

The PCNA index was assessed by point counting of positively stained nuclei present in at least 1000 malignant cells using a Nikon microscope (20× objective and 10× eyepiece) connected to a computer-assisted image analyser (Magiscan 2A, Joyce Loebl).

The patients in this study ranged in age from 25 to 64 years (median age 44 years). Of these, 46 patients had curative surgery and 4 had palliative surgery because of distant metastasis. Breast cancer recurred in 22 patients (44%) and 16 (32%) died due to

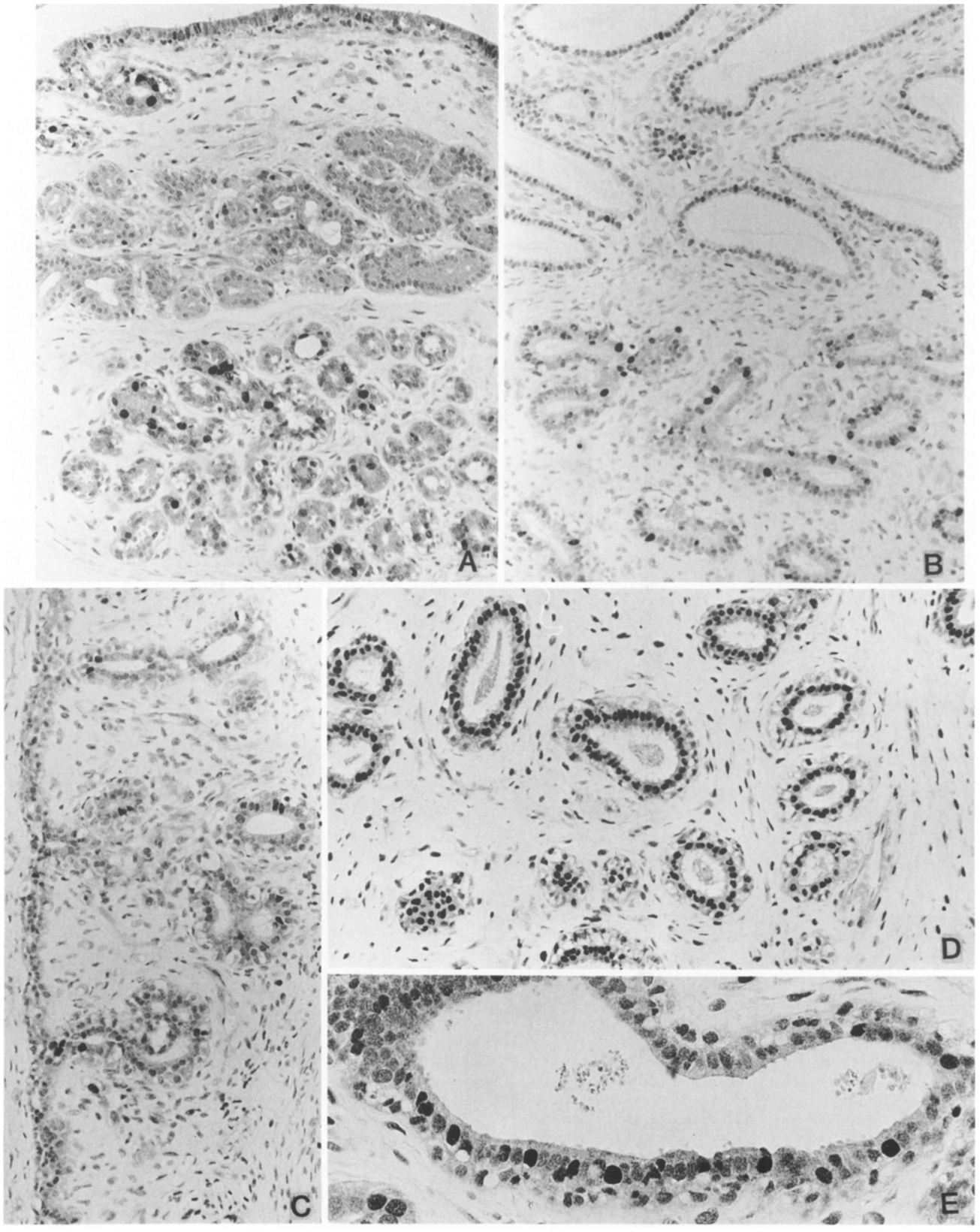


**Fig. 2 A–E.** PCNA immunostaining in fibroadenomas. **A** Fibroadenoma. There are two types of structure, small islands with epithelial proliferation and small tubular aggregates. Frequency of occurrence of PCNA-positive cells is 10.7%. **B** Multicystic structure composes of flattens epithelial cells. Frequency of PCNA-positive cells is 19.8% in this thin epithelial layer. **C** Frequency of PCNA-positive cells is 18.2% in both outer clear cells and luminal cells. **D** Fibroadenoma. Histopathology shows two cells layers consisting outer clear cells, modified myoepithelial compartment and luminal cells in dilated tubular structures. PCNA-positive cells are usually located in luminal cells. **E** Dilated duct-like structure of fibroadenoma. Frequencies of occurrence of PCNA-positive cells are 15.2% in luminal cells and 28.9% in outer cells. **A–D** ×100; **E** ×200

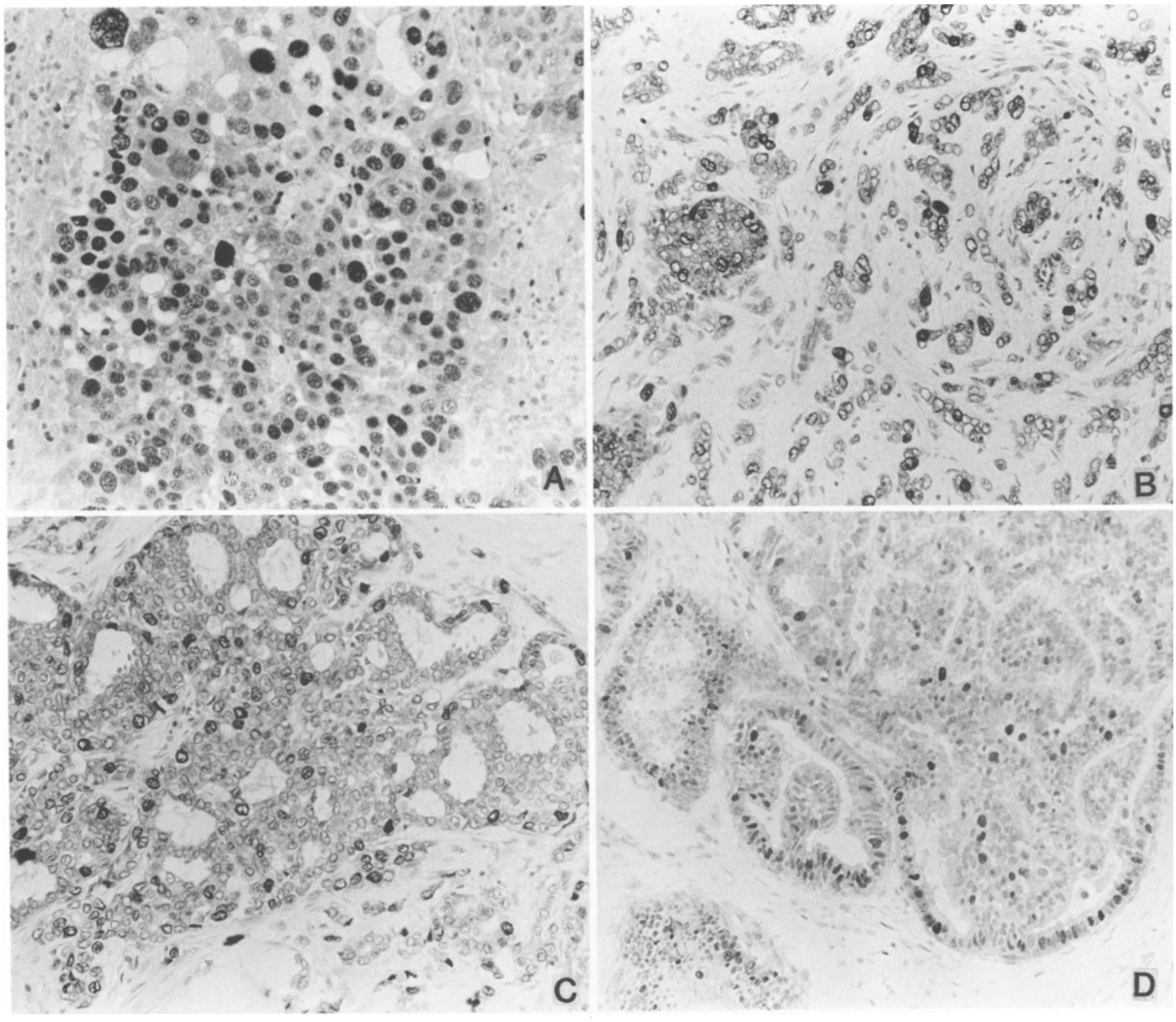


**Fig. 3A–D.** Carcinoma of the breast. **A** Solid tubular carcinoma shows high malignancy and almost all tumour cells express positive staining for PCNA. Frequency of PCNA-positive cells is 24%. **B** Infiltrating tumour cells of papillo-tubular carcinoma. Some of the scattered tumour cells show a positive PCNA reaction (frequency is 27%). **C** Papillo-tubular carcinoma. Positively staining cells scatter in tumour focus and frequency of PCNA-positive cells is 9.5%. **D** Papillo-tubular carcinoma. PCNA-positive tumour cells exist in the periphery of the tumour mass, and are scattered in the central area. Frequency of PCNA-positive cells is 8.2%. **A** ×200; **B–D** ×100

metastasis during the follow-up period. For all patients, follow-up data for periods longer than 60 months were available.

Association of proliferating cell index with c-*erb*B-2 overexpression, number of metastatic lymph nodes, tumour size on palpation, histopathological grade and clinical stage were tested for significance by using the chi-squared test. Curves for overall survival were drawn according to the Kaplan Meyer method.

## Results

*PCNA staining.* The normal and lactating breasts showed a low index of PCNA-positive nuclei (mean 3.6, SD 0.67) (Fig. 1A). The mean percentage of PCNA-positive nuclei in mastopathy was 23.7, SD 5.0 (Fig. 1B, C). Fibroadenomas showed small tubulo-ductal structures with two cell layers (Fig. 2A), tubular and cystic structures (Fig. 2B–E) and intercanalicular cellular proliferation, with the mean percentage of PCNA-positive cells 15.4, SD 3.1 in the epithelial cell components. In areas of multiple ductal structures, in 2 cases, luminal epithelial cells and irregular ductal cystic structures also showing to layers of cells, the luminal and outer modified myoepithelial cells showed a mean of 18.9%, SD 5.2% and 32.2%, SD 4.7% PCNA-positive cells respectively.

In primary breast carcinoma, the percentage of PCNA-positive nuclei ranged from 2% to 36% irrespective of the histopathological type (Fig. 3A–D). Tumours

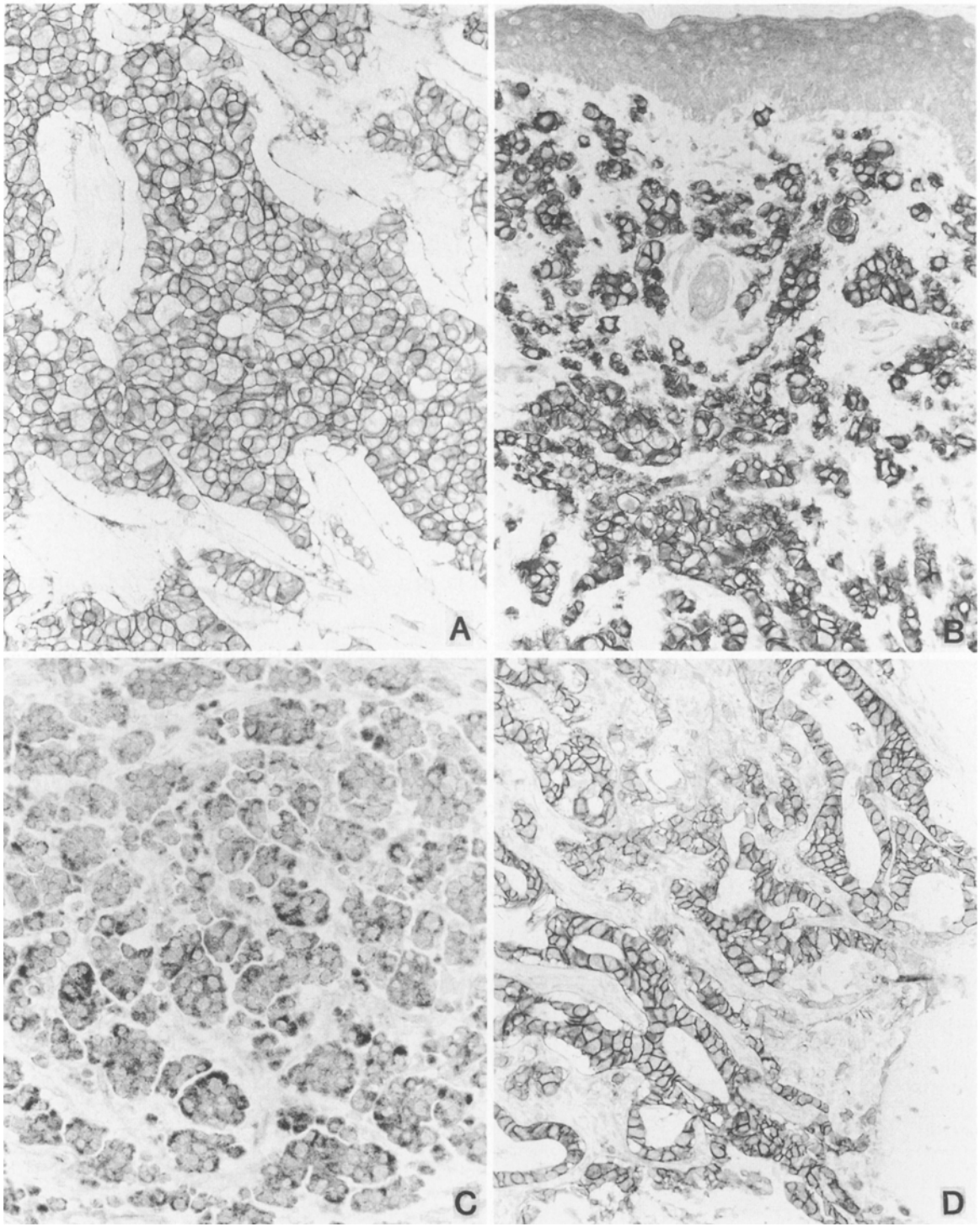


**Fig. 4 A–D.** c-*erb*-B-2 immunostaining in breast carcinoma. The sections only stain with c-*erb*B-2. No counterstaining. **A** Solid carcinoma shows the distribution of strong membrane positive type of c-*erb*B-2 staining. **B** Infiltrating carcinoma cells express strongly positive immunostaining in their neoplastic cells. **C** Liver metastasis of papillo-tubular carcinoma. Metastatic tumour cells show an intensely positive staining. **D** Bone metastasis. Metastatic papillo-tubular carcinoma shows cell membrane positive type. **A–D** $\times 100$

showing less than 5% (mean 3.6, SD 0.7, $n=18$), 6–10% (mean 8.0, SD 1.2, $n=10$) and more than 10% (mean 21.3, SD 6.5, $n=22$) PCNA-positive malignant cells were taken as having low, intermediate and high PCNA indexs respectively. Recurrent breast carcinoma showed a high PCNA index (mean 28.5, SD 4.0). The PCNA index was correlated with the c-*erb*B-2 protein and EGF receptor membrane reactivity and worsening histological grade. There was no significant association between the PCNA index and tumour size or node status or clinical stage. A high index was also associated with negative relation to survival and indicated better disease- free

**Table 1.** Proliferating cell nuclear antigen (PCNA) index and correlation with c-*erb*B-2 oncoprotein and epidermal growth factor (EGF) receptor expression

| | Low 5% | PCNA index Intermediate 5–10% | High 10% |
|---|---|---|---|
| c-*erb*B-2 Membrane reactivity* | | | |
| positive | 2 | 3 | 13 |
| negative | 16 | 7 | 9 |
| EGF receptor** | | | |
| positive | 1 | 5 | 16 |
| negative | 17 | 5 | 6 |

* $\chi^2 = 10.085$, $df = 2$, $P < 0.01$
** $\chi^2 = 18.066$, $df = 2$, $P < 0.005$

**Table 2.** PCNA index and clinicopathological variables

| | Low <5% | PCNA index Intermediate 5–10% | High >10% | Statistical significance |
|---|---|---|---|---|
| Tumor size | | | | |
| <5 cm | 13 | 6 | 15 | NS |
| >5 cm | 5 | 4 | 7 | |
| Lymph node status | | | | |
| negative | 9 | 4 | 12 | NS |
| positive | 9 | 6 | 10 | |
| Clinical stage | | | | |
| early | 12 | 7 | 12 | NS |
| advanced | 6 | 3 | 10 | |
| Histological grade | | | | |
| I | 10 | 3 | 5 | $\chi^2 = 9.776$ |
| II | 5 | 1 | 2 | $df = 2$ |
| III | 3 | 6 | 15 | $P < 0.05$ |

**Table 3.** c-*erb*B-2 status and clinicopathological variables

| | C-*erb*B-2 membrane immunoreactivity | | Statistical significance |
|---|---|---|---|
| | + ($n = 18$) | − ($n = 32$) | |
| Tumor size | | | |
| <5 cm | 10 | 24 | NS |
| >5 cm | 8 | 8 | |
| Lymph node status | | | |
| negative | 5 | 20 | $\chi^2 = 5.554$ |
| positive | 13 | 12 | $df = 1$, $P < 0.05$ |
| Clinical stage | | | |
| early | 7 | 24 | $\chi^2 = 6.375$ |
| advanced | 11 | 8 | $df = 1$, $P < 0.05$ |
| Histological grade | | | |
| I | 3 | 15 | $\chi^2 = 10.031$ |
| II | 1 | 7 | $df = 2$, $P < 0.01$ |
| III | 14 | 10 | |

**Table 4.** EGF receptor status and clinicopathological variable

| | EGF receptor membrane immunoreactivity | | Statistical significance |
|---|---|---|---|
| | + ($n = 22$) | − ($n = 28$) | |
| Tumour size | | | |
| <5 cm | 12 | 22 | $\chi^2 = 3.26$ |
| >5 cm | 10 | 6 | $df = 1$, NS |
| Lymph node status | | | |
| negative | 8 | 17 | NS |
| positive | 14 | 11 | |
| Clinical stage | | | |
| early | 10 | 21 | $\chi^2 = 3.814$ |
| advanced | 12 | 8 | $df = 1$, NS |
| Histological grade | | | |
| I | 2 | 16 | $\chi^2 = 23.169$ |
| II | 1 | 7 | $df = 2$, $P < 0.005$ |
| III | 19 | 5 | |

survival in patients with a low PCNA index (Tables 1, 2).

In c-*erb*B-2 staining 18 of the 50 patients with operable breast cancer showed positive membrane immunoreactivity with anti-c-*erb*B-2 polyclonal antibody (Fig. 4A–D). The benign tumours showed no membrane immunoreactivity; however, cytoplasmic staining present in a varying degree was not analysed further, as its significance is unknown. A highly significant correlation was found between poor survival and short relapse interval and tumour cell membrane staining. A significant correlation was also demonstrated between histological grade and membrane immunoreactivity, but no statistically significant correlation was observed between

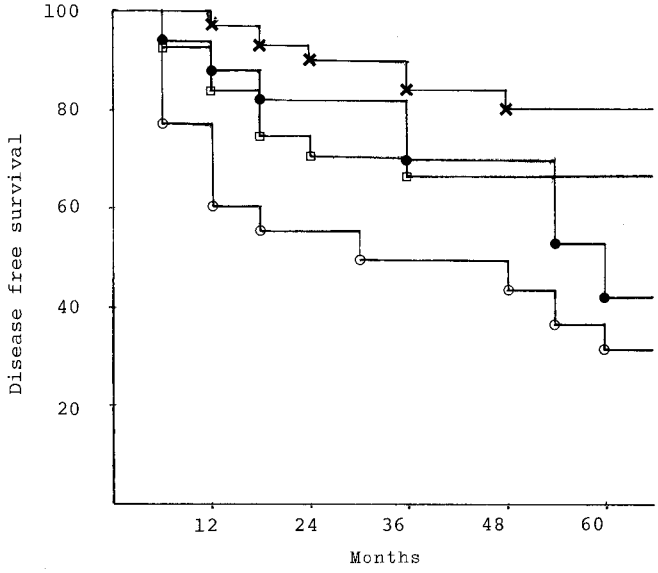


**Fig. 5.** Curves for overall survival in breast cancer patients with (●) and without (x) c-*erb*B-2 protein expression ($\chi^2 = 7.17$, $df = 1$, $P < 0.01$). Disease free survival in patients with (○) and without (□) c-*erb*B-2 protein expression. ($\chi^2 = 5.860$, $df = 1$, $P < 0.05$)

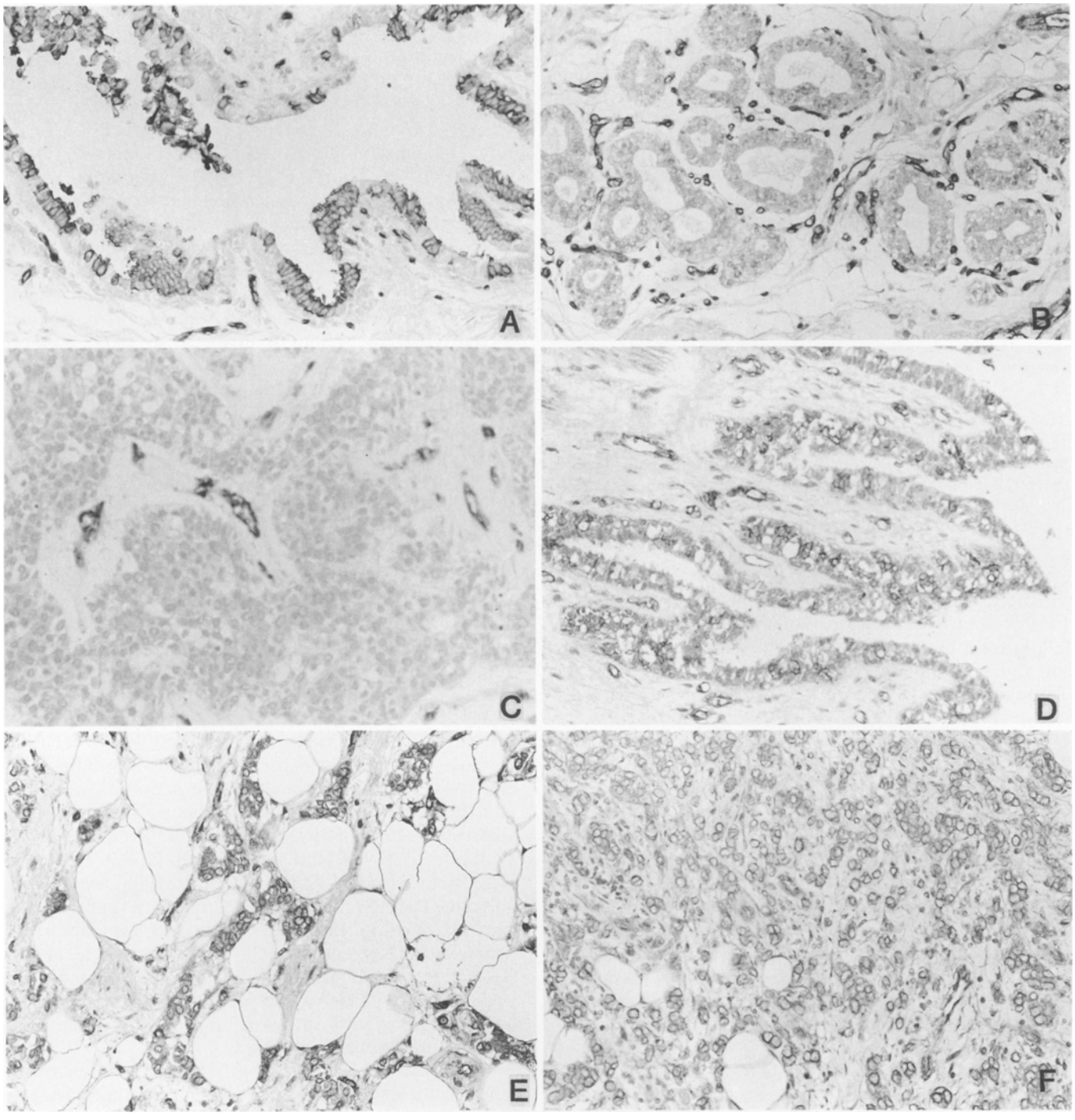


**Fig. 6A–F.** Epidermal growth factor (EGF) receptor immunostaining in breast lesions. The sections stain on EGF receptor and without counter staining. **A** Mastopathy. Epithelial cells of papillary projection in large cystic space express EGF receptor staining for cell membrane positive type. **C** Solid tubular carcinoma. Tumour cells stain positively for EGF receptor. **D** Papillary carcinoma. Carcinoma cells express the distribution of cell-membrane-positive type of EGF receptor staining. **E** Infiltrating carcinoma cells show strongly positive staining for EGF receptor. **F** Infiltrating tumour cells show cell-membrane-positive type for EGF receptor. The same specimen as Fig. 3B

tumour size, lymph node status and clinical stage (Table 3, Fig. 5).

In normal breast EGF receptor was only found on the luminal surfaces of the terminal ductule lobular unit. Fibroadenoma showed a negative EGF receptor reaction in ductal epithelial cells. In mastopathy, 78% of cases showed a surface border or focal cell membrane-positive immunoreactivity (Fig. 6A, B). In carcinoma, EGF receptor staining also showed cell membrane positive type or loss of staining in tumour cells (Fig. 6C–F). The pattern of EGF receptor immunoreactivity has been described in details (Wada et al. 1991).

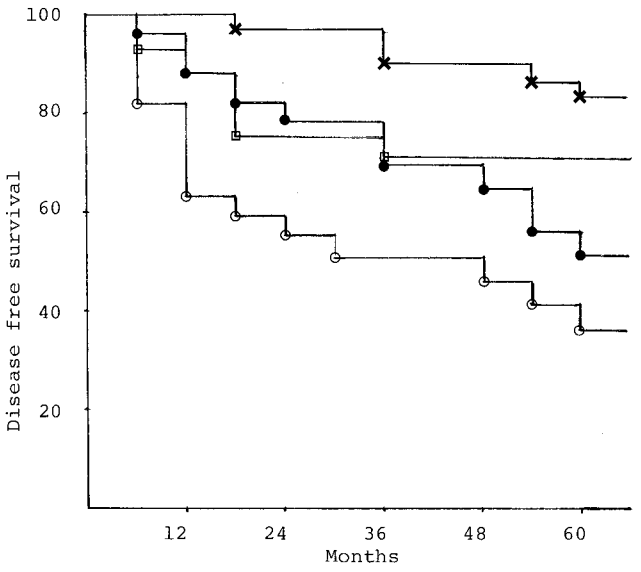


**Fig. 7.** Curves for overall survival in breast cancer patients with (●) and without (x) EGF receptor expression. ($\chi^2 = 5.849$, $df = 1$, $P < 0.05$). Disease free survival in patients with (○) and without (□) EGF receptor expression. ($\chi^2 = 6.147$, $df = 1$, $P < 0.05$)

There were 22 (44%) carcinomas with evidence of EGF receptor expression. A strong correlation ($P < 0.001$) was observed between the presence of EGF receptor and poor survival and shorter disease-free interval. Tumour size and lymph node status, however, showed no significant correlation with EGF receptor expression. Histological grade and clinical stage, however, was significantly associated with positive EGF receptor membrane reactivity (Table 4, Fig. 7).

## Discussion

The prognosis of patients presenting with operable breast cancer is extremely variable and tumour size, lymph node stage, histological grade and tumour type are currently recognized as the most powerful predictors of outcome (Todd et al. 1987). The 50 patients included in this series showed a statistically significant correlation between poor survival and tumour size ($P < 0.05$), nodal status ($P < 0.05$) and clinical stage ($P < 0.005$). Moreover, disease free survival was also correlated with the nodal status ($P < 0.005$) and clinical stage ($P < 0.01$) and a trend was observed with the size of the tumour ($\chi^2 = 3.25$, $df = 1$).

Cell kinetic information has been regarded as a potential prognostic indicator and the purpose of this study was to establish the proliferating fraction of tumour cells in terms of clinical outcome. The significance of the expression of the proto-oncogene c-*erb*B-2 and EGF receptor, as well as other tumour variables in patients presenting with primary breast cancer, was also studied.

As the c-*erb*B-2 oncogene protein has a structure highly homologous to that of EGF receptor, it is most likely that overexpression of the protein may be associated with rapid tumour cell turnover. In the present study a statistically significant association was observed between EGF receptor and c-*erb*B-2 expression and the fraction of tumour cells in S-phase as determined on the basis of PCNA immunoreactivity. Previous reports have noted that the proliferative activity in breast cancer measured by thymidine incorporation into DNA or by flow cytometry is related to histological features (Dawson et al. 1990; Thorud et al. 1986). In our study, a high rate of proliferation was accompanied by nuclear anaplasia and the histological dedifferentiation. A high index of proliferation determined on the basis of PCNA immunohistochemistry was correlated with histological grade; however, it showed no relationship to tumour size or nodal involvement. PCNA index was significantly higher in the recurrent tumour than in the primary, possibly related to aggresive behaviour. Moreover, a high PCNA index was associated with poor prognosis in terms of actual and disease-free interval survival. A high S-phase fraction has been shown to be an indicator of poor prognosis in breast cancer (O'Reilly et al. 1990a, b), and PCNA staining may be an excellent marker for S-phase DNA content in cells (van Dierendonck et al. 1991).

Previous studies have confirmed the relationship between c-*erb*B-2 gene amplification and immunohistochemical detection of the oncogene protein product (van de Vijver et al. 1987; Venter et al. 1987). We found that the c-*erb*B-2 oncoprotein was expressed in 36% of the cancer patients. This level of expression ranged from 10% to 30% as reported in the previous literature (Ali et al. 1988; Slamon et al. 1987). The overall survival and relapse-free survival for patients with tumours positive for c-*erb*B-2 oncoprotein was significantly poorer than in those tumours not expressing the oncoprotein. This effect was observed throughout the period of follow-up and these findings are similar to those observed by Lovekin et al. (1989), Paik et al. (1990), Tsuda et al. (1989), Walker et al. (1989) and Wright et al. (1989). However, Barnes et al. (1988), Gusterson et al. (1988) and van de Vijver et al. (1988) have reported no statistically significant relationship, although trends hve been observed. Moreover, Slamon et al. (1989) and Tandon et al. (1989) have reported no association with poor prognosis in node negative breast cancer. The present study shows a statistically significant association in both node-positive and node-negative patients.

A statistically significant difference has been noted between the expression of c-*erb*B-2 and other variables such as nodal involvement and histological grade. In this study 38% of the tumours were stage III and IV tumours and 50% were from patients without lymph node involvement. A correlation beteen c-*erb*B-2 amplification and lymph node status has been suggested by Guerin et al. (1989) but not by Slamon et al. (1989), Tandon et al. (1989), Tsuda et al. (1989), van de Vijver et al. (1988), Walker et al. (1989) and Wright et al. (1989).

The mechanism by which the c-*erb*B-2 oncogene induces malignant change is not yet known. However, 2 patients who had a consistently c-*erb*B-2 negative grade I primary breast cancer with low PCNA index were found to have c-*erb*B-2-positive, grade III cancers with

PCNA index when the tumours recurred. Both the primary tumours were positive for EGF receptor expression. With such a relatively small number co-expressing c-*erb*B-2 protein statistical analysis is unpossible. Six of our c-*erb*B-2 positive primary breast cancer patients have a disease-free survival of 6–9 years. This underlines the importance of studying large numbers of patients when evaluating prognosis.

The EGF receptor immunoreactivity in previously reported series ranges from 14% to 43% (Hawkins et al. 1991; Lewis et al. 1990; Lundy et al. 1991). A positive correlation with poor survival and poor disease-free survival have been suggested by Sainsbury et al. (1987) and Lewis et al. (1990). Conflicting variation has been reported regarding the association between EGF receptor status and other clinicopathological variables (Lewis et al. 1990; Lundy et al. 1991).

The present study shows that PCNA index, c-*erb*B-2 and EGF receptor status are independent variables for predicting the clinical outcome, although c-*erb*B-2 positive tumours tend to be EGF receptor positive with high PCNA index. We suggest that c-*erb*B-2 and EGF receptor expression combined with PCNA index provide a superior means of distinguishing between patients in the high risk group who are subject to early relapse of the disease than using only c-*erb*B-2 or EGF receptors. This discrimination would be of potential therapeutic significance, as a group of patients with a poor prognosis may be identified in which systemic adjuvant therapy (endocrine manipulation or chemotherapy) is justified. However, this should be confirmed by a prospective study in a large group of patients.

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