Virchows Archiv A Pathol Anat (1992) 420:345–351

Virchows Archiv A
Pathological Anatomy and Histopathology
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Immunohistochemical studies on oncogene products (EGF-R, c-*erb*B-2) and growth factors (EGF, TGF-α) in human breast cancer: their relationship to oestrogen receptor status, histological grade, mitotic index and nodal status

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Received August 13, 1991 / Received after revision December 4, 1991 / Accepted December 5, 1991

Summary. In this investigation, 83 human mammary carcinomas were examined for the expression of oestrogen receptor (ER), epidermal growth factor receptor (EGF-R), epidermal growth factor (EGF), transforming growth factor alpha (TGF-α), c-*erb*B-2, histological grade, mitotic index and nodal status, all of which are reportedly prognostically significant factors (Bloom and Richardson 1957; Baak et al. 1985; Wright et al. 1989). ER expression was biochemically recognized in 43.4% of mammary carcinomas, and EGF-R, EGF, TGF-α and c-*erb*B-2 were histochemically recognized in 25.3, 14.5, 27.7 and 18.0% of mammary carcinomas examined respectively, using conventional sections of buffered formalin-fixed, paraffin-embedded tissue and monoclonal or polyclonal antibodies. There were significant relationships between negative ER and positive EGF-R or TGF-α; positive EGF-R and TGF-α; positive EGF-R and c-*erb*B-2; and positive c-*erb*B-2 and TGF-α. The single changes which were the negative ER and the positive c-*erb*B-2 correlated with histological grade and mitotic index. Co-expression of EGF-R and TGF-α correlated with positive nodal status. Therefore, the present investigation indicates that the negative ER, single expression of c-*erb*B-2 and co-expression of EGF-R and TGF-α are important markers which contribute indirectly to prognosis, which reconfirms previous findings on the former two while adding the new finding that immunohistochemical demonstration of expression of EGF-R and TGF-α may provide useful information for selecting the appropriate treatment.

Key words: Breast cancer – Epidermal growth factor – EGF receptor – Transforming growth factor-α – c-*erb*B-2

Introduction

Only about 50% of all patients presenting with primary breast cancer are cured by local therapy. It would therefore be desirable to the able to predict which patients are likely to have a recurrence so that systematic therapy can be instituted to delay, or even prevent such recurrences. Recently it has been confirmed that breast cancer cells can synthesize and secrete various growth factors and the receptors for them may stimulate tumour growth through autocrine and/or paracrine mechanisms (Osborne and Artega 1990). The expression of certain oncogenes, growth factor receptors or the growth factors themselves in human breast cancers have been studied with regard to their biological characteristics. Oestrogen receptors (ER) are well-known prognostic indicators (Knight et al. 1977) and mammary tumours rich in ER are more likely to respond to endocrine therapy (Roberts et al. 1978). Epidermal growth factor receptors (EGF-R) on human breast cancer cells have been described both in some derived cell lines and resected specimens. Epidermal growth factor (EGF) is a polypeptide that influences proliferation, differentiation and functional activities of various types of cells containing mammary cells in vivo and in vitro (Taylor et al. 1972; Okamoto and Oka 1984). EGF gene mRNA is expressed in a large proportion of human breast cancer biopsy samples (Dotzlaw et al. 1990). Transforming growth factor alpha (TGF-α) is an acid and heat stable protein of molecular weight 5.5 kDa (Derynck 1986), which is 33% homologous at the amino acid level to EGF (Marquardt et al. 1983). Some breast cancer cells produce TGF-α which acts by way of the EGF-R (Reynolds et al. 1983; Dickson et al. 1986). Therefore, an autocrine self-stimulatory role has been postulated (Sporn and Todaro 1980). There is a highly significant inverse relationship between expression of EGF-R and ER, and the presence of EGF-R is associated with higher Bloom and Richardson's histological grades and decreased patient survival (Sainsbury et al. 1987a, b; Spyratos et al. 1990). The c-*erb*B-2 protein is similar in structure to EGF-R (its amino acid sequence is about 50% homologous to that of EGF-R but its functions and ligands remain unknown; King et al. 1985; Schechter et al. 1985; Semba et al. 1985). Since the first report on the association between c-*erb*B-2

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gene amplification and unfavourable disease outcome or poor prognosis in human breast cancer by Slamon et al. (1987), several studies on the subject have been published by various investigators (Berger et al. 1988; Jungsil et al. 1989; Thor et al. 1989; Tsuda et al. 1989; Wright et al. 1989; Yamada et al. 1989; Borg et al. 1990; Uehara et al. 1990). However, there is controversy over the incidence and clinical significance of c-*erb*B-2 amplification in breast cancer (Ali et al. 1988; Gusterson et al. 1988; Vijver et al. 1988; Meyers et al. 1990). In the investigation reported here, we examined the expression of growth factors, growth factor receptors and oncogene products, using immunohistochemical methods in conventional sections of formalin-fixed, paraffin-embedded tissue from breast cancer, which has the advantage of obtaining findings more promptly than biochemical methods. Our purpose was to examine whether or not positive staining is a marker which can contribute indirectly to the biological characteristics of breast cancer and whether or not this method would provide useful information concerning proper therapy.

Materials and methods

Primary breast cancers and axillary lymph nodes were resected surgically from 83 Japanese female patients consecutively admitted to Sagara Hospital between November 1989 and April 1991. Their ages ranged from 34 to 81 years, with a median of 52.2 years. A major part of each tumour was used for histological studies, and the remainder for ER determinations.

Pieces of tumour and axillary lymph nodes were fixed in 10% neutrally buffered formalin, and embedded in paraffin. Sections 3 μm thick were stained with haematoxylin and eosin, and were examined histopathologically. The mitotic index was defined as the total number of mitosis in ten high-power fields (400 × magnification) as previously described (Baak et al. 1985). Breast cancer was classified histologically according to the criteria of the World Health Organization (WHO). Histological grade was determined using a modification of the method of Bloom and Richardson (1957).

Avidin-biotin complex immunoperoxidase assays were performed with the Vectastain ABC kit (Vector Laboratories, Burlingame, Calif., USA). Deparaffinized and rehydrated sections were treated with 0.3% hydrogen peroxide in methanol solution to quench endogenous peroxidase activity and were subsequently incubated at room temperature with the following reagents (with phosphate-buffered saline washes in between): 1.0% normal bovine serum albumin (BSA) for 30 min, primary antiserum diluted in PBS with 1.0% BSA (EGF-R; 1:50, EGF; 1:50, TGF-α; 1:100, c-*erb*B-2; 1:10) for 30 min biotinylated goat anti-rabbit or mouse antiserum for 30 min, ABC complex for 30 min, and 3,3′-diaminobenzidine tetrachloride containing 0.05% hydrogen peroxide for 5 min. The sections were then washed, counterstained, dehydrated, cleared in xylene, and mounted. Controls were set up with phosphate buffered saline containing 1.0% normal BSA instead of the primary antisera.

Staining of the cytoplasmic membrane was estimated to be a positive expression of c-*erb*B-2 and staining of cytoplasm was considered to be positive expression of EGF-R, EGF and TGF-α, as previously described (Dazzi et al. 1989; Uehara et al. 1990).

The c-*erb*B-2 antibody (pAb1) purchased from Triton Bioscience (Alameda, Calif., USA) was a rabbit antibody raised against a synthetic peptide corresponding to a cytoplasmic epitope of the c-*erb*B-2 protein conjugated to keyhole limpet haemocyanin. The specificity of the antibody was proven by Western blot analysis (Gullick et al. 1987). The EGF-R (Ab-4) purchased from Oncogene Science (Manhasse, N.Y., USA) is a rabbit, affinity-purified polyclonal antibody raised against a peptide (D V V D A D A D E Y L I P Q), which corresponds to amino acid residues 1005 to 1016 (Gullick et al. 1985). TGF-α (Ab-2) purchased from Oncogene Science was derived by immunizing BALB/c mice with recombinant human TGF-α and fusing mouse splenocytes with P3 × 63 Ag8·653 mouse myeloma cells (Sorvillo et al. 1990). Monoclonal antibody to human EGF purchased from Wakunaga Pharmaceuticals (Hiroshima, Japan), was derived by immunizing Balb/c mice with recombinant human EGF and fusing mouse splenocytes with P3U1 myeloma cells.

ER levels were determined using dextran-coated charcoal (Biomedical Laboratories, Tokyo, Japan). A concentration greater than 14 fmol ER/mg of protein was considered positive. For statistical analysis, the association of five expressed proteins with other categorized clinicopathological variables such as tumour size, nodal status, TNM stage, histological stage and mitotic index or among the expressed proteins themselves was assessed by chi-square analysis.

Table 1. Expression of oestrogen receptor (ER), epidermal growth factor-receptor (EGF-R), epidermal growth factor (EGF), transforming growth factor alpha (TGF-α) and c-*erb*B-2

ER	EGF-R	EGF	TGF-α	c-*erb*B-2	Total no. (%)
+	−	−	−	−	25 (30.1%)
+	−	−	+	−	1 (1.2%)
+	−	−	−	+	3 (3.6%)
+	−	+	−	−	2 (2.4%)
+	+	−	+	−	2 (2.4%)
+	+	+	−	−	1 (1.2%)
+	−	−	+	+	1 (1.2%)
+	−	+	+	−	1 (1.2%)
−	−	−	−	−	19 (22.9%)
−	+	−	−	−	3 (3.6%)
−	−	−	+	−	2 (2.4%)
−	−	−	−	+	3 (3.6%)
−	−	+	−	−	3 (3.6%)
−	+	−	+	−	4 (4.8%)
−	+	−	−	+	1 (1.2%)
−	−	+	+	−	2 (2.4%)
−	+	−	+	+	7 (8.4%)
−	+	+	+	−	3 (3.6%)
36 (43.4%)	21 (25.3%)	12 (14.5%)	23 (27.7%)	15 (18%)	83

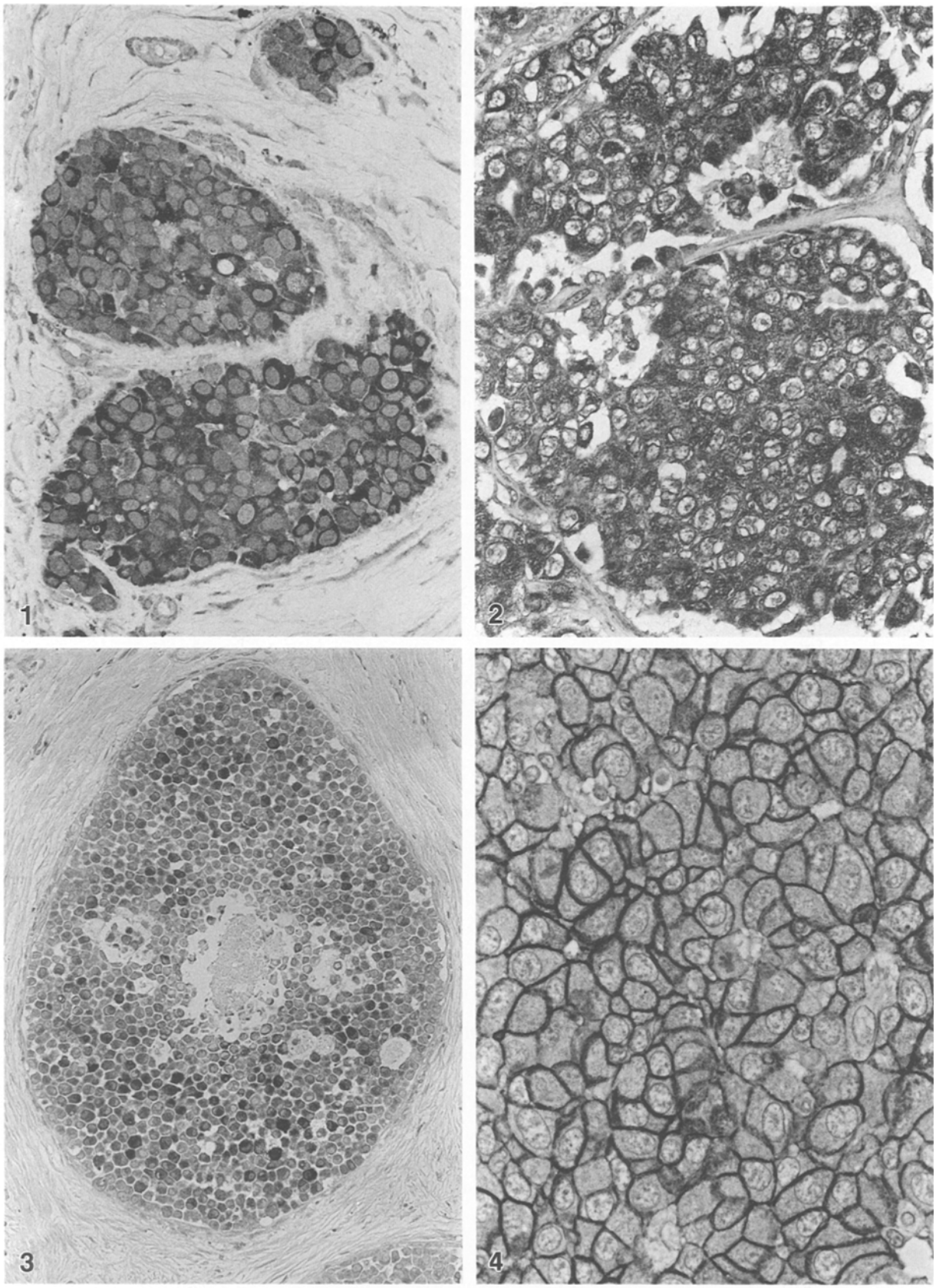

Fig. 1. Positive epidermal growth factor-receptor staining in cytoplasm of mammary carcinoma cells. ABC method, ×217

Fig. 2. Positive epidermal growth factor staining in cytoplasm of mammary carcinoma cells. ABC method, ×330

Fig. 3. Positive transforming growth factor alpha staining in cytoplasm of mammary carcinoma cells. ABC method, ×132

Fig. 4. Positive c-*erb*B-2 staining at cytoplasmic membranes of mammary carcinoma cells. ABC method, ×528

Results

Of the 83 invasive carcinomas in this study, there were 73 ductal, 5 medullary, 2 mucinous, 2 lobular and 1 apocrine. Expression of ER and positive staining in mammary carcinomas examined in the present investigations is summarized in Table 1. ER, EGF-R (Fig. 1), EGF (Fig. 2), TGF-α (Fig. 3), and c-*erb*B-2 (Fig. 4) were recognized in 43.4, 25.3, 14.5, 27.7 and 18.0%, respectively, of mammary carcinomas. There was a significant correlation between negative ER and positive EGF-R or TGF-α staining; positive EGF-R and TGF-α staining; positive EGF-R and c-*erb*B-2 staining and positive c-*erb*B-2 and TGF-α staining (Table 2). The single change of negative ER or positive c-*erb*B-2 staining was significantly correlated with histological grade and mitotic index, but not with nodal status (Tables 3, 7). Positive staining of EGF-R, EGF or TGF-α was not significantly associated with variables such as the size of tumours, nodal status, TNM stage, histological grade or

Table 2. Relationship between ER, EGF-R, EGF, TGF-α and c-*erb*B-2 expression

Observations compared	*P*
ER vs EGF-R	<0.01[a]
ER vs TGF-α	<0.05[a]
ER vs c-*erb*B-2	NS
ER vs EGF	NS
EGF-R vs TGF-α	<0.00001
EGF-R vs c-*erb*B-2	<0.025
TGF-α vs c-*erb*B-2	<0.05
EGF vs EGF-R	NS
EGF vs c-*erb*B-2	NS
EGF vs TGF-α	NS

NS, Not significant; [a] inverse relationship

Table 3. Association between ER expression and various disease finding

	36 tumours with expression	47 tumours without expression	*P*
Tumor size (mm)			
0–20	4	7	NS
21–50	21	28	
51–100	11	12	
Lymph node metastasis			
positive	22	23	NS
negative	14	24	
TNM stage			
I and II	21	28	NS
III and IV	15	19	
Histologic grade			
I	16	10	<0.005
II	17	19	
III	3	18	
Mitotic index			
0–9	25	22	<0.005
10–20	8	5	
>20	3	20	

NS, Not significant

Table 4. Association between EGF-R expression and various disease findings

	21 tumours with expression	62 tumours without expression	*P*
Tumor size (mm)			
0–20	3	4	NS
21–50	9	39	
51–100	9	19	
Lymph node metastasis			
positive	14	31	NS
negative	7	31	
TNM stage			
I and II	10	43	NS
III and IV	11	19	
Histological grade			
I	6	21	NS
II	7	29	
III	8	12	
Mitotic index			
0–9	9	38	NS
10–20	3	9	
>20	9	15	

NS, Not significant

Table 5. Association between EGF expression and various disease findings

	12 tumours with expression	71 tumours without expression	*P*
Tumor size (mm)			
0–20	2	10	NS
21–50	5	42	
51–100	5	19	
Lymph node metastasis			
positive	5	40	NS
negative	7	31	
TNM stage			
I and II	5	41	NS
III and IV	7	30	
Histologic grade			
I	5	15	NS
II	5	33	
III	2	23	
Mitotic index			
0–9	8	39	NS
10–20	2	10	
>20	2	22	

NS, Not significant

Table 6. Association between TGF-α expression and various disease findings

	23 tumours with expression	60 tumours without expression	*P*
Tumor size (mm)			
0–20	3	3	NS
21–50	11	37	
51–100	9	20	
Lymph node metastasis			
positive	14	31	NS
negative	9	29	
TNM stage			
I and II	11	39	NS
III and IV	12	21	
Histological grade			
I	9	18	NS
II	7	29	
III	7	13	
Mitotic index			
0–9	12	35	NS
10–20	2	10	
>20	9	15	

NS, Not significant

Table 7. Association between c-*erb*B-2 expression and various disease findings

	15 tumours with expression	68 tumours without expression	*P*
Tumor size (mm)			
0–20	1	7	NS
21–50	7	40	
51–100	7	21	
Lymph node metastasis			
positive	11	34	NS
negative	4	34	
TNM stage			
I and II	7	45	NS
III and IV	8	23	
Histological grade			
I	0	25	<0.005
II	6	32	
III	9	11	
Mitotic index			
0–9	4	44	<0.025
10–20	3	8	
>20	8	16	

NS, Not significant

mitotic index (Tables 4–6). Concurrent positive staining of EGF-R and TGF-α was significantly correlated with nodal status (Table 8). Co-expression of other receptors, growth factors and the oncogene product examined in the present investigation did not significantly correlate with these variables.

Table 8. Association between various disease findings and co-expression of EGF-R and TGF-α

	EGF-R (+) TGF-α (+)	EGF-R (−) TGF-α (−)	*P*
Tumor size (mm)			
0–20	3	10	NS
21–50	8	31	
51–100	5	14	
Lymph node metastasis			
positive	13	26	<0.05
negative	3	29	
TNM stage			
I and II	8	36	NS
III and IV	8	19	
Histological grade			
I	5	15	NS
II	6	27	
III	5	13	
Mitotic index			
0–9	8	34	NS
10–20	1	8	
>20	7	13	

NS, Not significant

Discussion

This is the first report of an immunohistochemical examination of EGF-R, EGF, TGF-α and c-*erb*B-2 expression in conventional sections of formalin-fixed, paraffin-embedded mammary carcinomas from a large number of patients, in which histological grade, mitotic index and nodal status have been compared. In the present study, the percentage of ER-positive mammary carcinomas was 43.4% of all mammary carcinomas examined. The incidence is slightly lower than that of previous study on mammary carcinomas in Japan (Matsumoto et al. 1978). The lower incidence of ER positive cases seems to be due to the relatively advanced stage of the mammary carcinomas examined in the present study; this arose because biochemical and histochemical examinations needed a relatively large amount of tumour tissue. The positive immunohistochemical staining obtained in the present examination is thought to reflect over-expression of EGF-R (Gullick et al. 1986; Real et al. 1986; Spyratos et al. 1990). EGF (Dotzlaw et al. 1990) and TGF-α (McGuire et al. 1988; Mizukami et al. 1990) and correlates with the gene amplification of c-*erb*B-2 (Venter et al. 1987; Gusterson et al. 1988; Uehara et al. 1990), according to previous studies. The incidence of mammary carcinomas with c-*erb*B-2 immunoreactivity examined in the present investigation was similar to that reported in previous studies (Gusterson et al. 1988; Wright et al. 1989; Uehara et al. 1990). However, the

incidence of those with EGF-R or TGF-α is lower, compared with previous studies (Sainsbury et al. 1987; Mizukami et al. 1990). Therefore, the low incidence may be peculiar to breast cancer in the Kagoshima area of southern Japan, where the death rate per 100,000 was 7.9 in 1989, which was lower than that of other areas of Japan. Further biochemical studies are necessary, however, in order to determine whether the low incidence is due to artificial changes occurring during paraffin embedding or not. In the present investigation using an immunohistochemical method, positive staining of EGF was observed in only 14.5% of breast cancer specimens examined, although a biochemical study has shown that EGF mRNA is detectable in 83% of breast cancer biopsy samples (Dotzlaw et al. 1990). Immunohistochemical methods using the same antibody for EGF can reveal positive staining in salivary gland cells. Therefore, the results in the present and previous investigations suggest that the ratio of breast cancers with high levels of EGF may be low, although a further biochemical study on post-translational system and degradation or secretion of EGF in tumour cells is necessary. The finding of an inverse relationship between ER and EGF-R expression in the present investigation agrees with those of previous studies (Sainsbury et al. 1987a, b). The results of this study show an inverse relationship between the expression of ER and TGF-α and co-expression of EGF-R and TGF-α, co-expression of EGF-R and c-*erb*B-2, and co-expression of c-*erb*B-2 and TGF-α. Single changes which were significantly related to clinicopathological variables were negative ER and the single expression of c-*erb*B-2, which correlated with histological grades and mitotic index. The changes which correlated with nodal status was co-expression of EGF-R and TGF-α. There are many reports indicating that histological grade, mitotic index and metastasis to lymph nodes are prognostic factors (Bloom et al. 1957; Baak et al. 1985; Thor et al. 1989; Wright et al. 1989). This study indicates that the negative ER, single expression of c-*erb*B-2 and co-expression of EGF-R and TGF-α are important markers which contribute indirectly to prognosis, which reconfirms previous findings on the former two (Knight et al. 1977; Slamon et al. 1987; Berger et al. 1988; Jungsil et al. 1989; Wright et al. 1989; Borg et al. 1990; Uehara et al. 1990) while adding the new finding that co-expression of EGF-R and TGF-α should be a useful indicator of prognosis.

Surgical treatment of breast cancer is changing from aggressive Halsted, Patey or Auchincloss methods to limited lumpectomy, quadrantectomy or simple mastectomy. It is important for surgeons to be able to give a pre-operative prognosis especially with regard to lymph node metastases, in order to determine the appropriate therapeutic strategy. The present immunohistochemical investigation showed that expression of EGF-R, TGF-α and c-*erb*B-2 in biopsy specimens, in addition to the assessment of the status of ER, may provide preoperative prediction of the biological behaviour of some carcinomas.

Acknowledgements. We are grateful to T. Kodama and T. Nitanda for their technical assistance. This work was supported, in part, by the Kodama Memorial Fund for Medical Research.

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