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Immunohistochemical studies on oncogene products (c-erbB-2, EGFR, c-myc) and estrogen receptor in benign and malignant breast lesions

With special reference to their prognostic significance in carcinoma

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Summary. It is a matter of debate whether the amplification of c-erbB-2 oncogene or production of the oncoprotein in breast cancers correlate with the presence of lymph node metastasis and with a poor prognosis. This study was aimed at elucidating the immunohistochemical localization of oncogene products which are related to cell growth, c-erbB-2 product, epidermal growth factor receptor (EGFR), c-myc protein and estrogen receptor (ER), in benign and malignant lesions of the breast. Fresh frozen sections of 25 breast cancers and 11 fibroadenomas from Japanese women were studied by indirect immunoperoxidase method with proper fixation. C-erbB-2 product and EGFR were localized on the cell membrane whereas c-myc protein and ER were observed in the nuclei. Immunohistochemical expression of oncogene products and ER were not only observed in the mammary carcinomas but also in the fibroadenomas. However immunoreactivities of EGFR and ER were more frequently seen in the fibroadenomas ($p < 0.05$). In breast cancers, the incidence of immunoreactivity for c-erbB-2 was higher in the cases with lymph node metastasis than cases without nodal metastasis ($p < 0.05$) and there was reciprocal correlation between the expressions of EGFR and ER ($p < 0.05$). Regarding the size of the primary tumour, there was no statistically significant correlation with the expressions of c-erbB-2, EGFR, c-myc or ER. Histological grade correlated only with the expression of ER ($p < 0.05$).

Key words: Breast tumours – Oncogene products – Immunohistochemistry – Lymph node metastasis

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Introduction

Recently there have been many reports about expression of oncogenes and oncogene products in human breast cancers, with regard to their relationship to lymph node metastasis, primary tumour size, poor prognosis, and histological grading (Sainsbury et al. 1987; Slamon et al. 1987; Varley et al. 1987; Zhou et al. 1987; Berger et al. 1988; Vijver et al. 1988; Wright et al. 1989). In particular c-erbB-2, also known as neu oncogene, HER-2 and MAC117, which is a 185 kDa transmembrane glycoprotein with tyrosine kinase activity coded in human chromosome 17 at q21 (Schechter 1984; Coussens 1985; King 1985; Semba 1985; Akiyama 1986; Bargmann 1986; Yamamoto 1986) are considered to be independent prognostic indicators in primary breast cancer (Slamon et al. 1987; Berger et al. 1988; Vijver et al. 1988; Wright et al. 1989). The relationship of c-erbB-2 and lymph node metastasis is one of the most important prognostic factors and the oncogene has been demonstrated to be amplified by dot blot analysis (Slamon et al. 1987) and to be overexpressed by immunohistochemistry using paraffin-embedded tissues (Berger et al. 1988). This has been questioned by other investigators using paraffin embedded tissues (Vijver et al. 1988; Wright et al. 1989). Our current immunohistochemical studies are aimed at to elucidate the expressions of c-erbB-2 product, epidermal growth factor receptor (EGFR), c-myc protein and estrogen receptor (ER) in benign and malignant breast lesions using fresh frozen tissues. Their significance as prognostic indicators is also investigated, especially in term of their relationship to lymph node metastasis.

Material and method

Thirty six surgically resected breast tumours (25 malignant, 11 benign) from Japanese female were collected in the Division of Diagnostic Pathology, Tokai University Hospital, Isehara, Japan. Their sources and pathological diagnosis are shown in Tables 1 and 2. All benign tumours were fibroadenomas. In malignant tumours, average age was 52.8±13.5 and the mean of the maximum tumour diameter was 3.2±1.7. There were 14 cases with lymph node metastasis and 11 cases without nodal metastasis. All materials were immediately frozen in O.C.T. compound and kept frozen for immunohistochemical study at −70° C. The cancer tissue was snap-frozen and stored in liquid nitrogen before transportation in dry ice for biochemical determination of estrogen receptors.

Histological grade was determined using a modification of Bloom and Richardson's method (Elson et al. 1982).

Sections for immunohistochemistry were cut at 6 μm and were fixed in proper fixatives at room temperature for 30 min. The list of antibodies and fixatives used is shown in Table 3. The time for incubation and rinsing of antibodies was 30 min. The sites of peroxidase reactivity were visualized by 25 mg% diaminobenzidine Tris-HCl solution, pH 7.6. The sections were counterstained with 5% methylgreen, pH 4.0. Immunoreactivities were divided into six groups from negative (−) to strongly positive (++++) according to number of positive cells as detailed in footnote of Table 1. However, staining intensity varied from one antibody to the other; the intensity of c-erbB-2, c-myc and ER was stronger than that of EGFR.

Table 2. Immunohistochemistry of fibroadenomas

Case	c-erbB-2	EGFR	c-myc	ER
1	++	+	+++	++
2	+	+	+++	++
3	+	+	+++	+
4	+	+	−	++
5	−	+	±	+
6	++	±	+++	++
7	++	+++	+++	+
8	+++	±	+++	+
9	++	+	+++	+
10	++	++	+++	+
11	+++	±	+++	++

Histological grading and immunoreactive cell count were carried out independently by two pathologists, and a consensus on discrepant cases was reached by reassessment on a double-headed microscope.

Biochemical determination of estrogen receptor was performed according to the dextran-coated charcoal (DCC) method and single point titration assay (measurements done by Teijin Bioscience Laboratory, Japan) (McGuire et al. 1977; Johnson and Nakamura 1978; Namkung and Petra 1981). A concentration greater than 3 fmol of ER per milligram of protein was considered to be positive.

Table 1. Details of the cases and immunohistochemistry of breast cancers

Case	Age	Histology		Grade[2]	Size (cm)	LN meta	c-erbB-2	EGFR	c-myc	ER	ER (DCC[3])
		WHO	Japan MCS[1]								
1	43	IDC[4]	S-T[7]	III	3.5×3.5	0/5	++	−	+++	+++	40
2	53	IDC	Sci[8]	II	3.5×2.5	2/20	+++	−	+++	−	LT3[10]
3	56	IDC	S-T	II	5.0×4.5	0/22	−	−	+++	−	LT3
4	70	IDC	Sci	I	1.5×1.5	0/16	±	−	++++	+++	176
5	33	SQ[5]	SQ	III	1.6×1.2	1/21	+++	+++	±	−	ND[11]
6	67	IDC	Sci	I	1.5×1.2	0/18	+	−	+++	+++	231
7	72	Muc[6]	Muc	I	2.0×2.0	0/22	+	+	+	+++	95
8	48	IDC	S-T	II	5.5×5.0	44/48	+++	+++	+++	−	LT3
9	43	IDC	Sci	I	4.5×3.5	10/17	++++	−	+++	++	50
10	49	IDC	S-T	I	2.0×2.0	1/14	+++	−	+++	+++	LT3
11	51	IDC	S-T	II	5.0×4.0	0/40	±	+++	+	++	38
12	72	IDC	Sci	I	6.0×5.0	0/25	+	−	+++	++	50
13	43	IDC	Sci	III	4.2×3.2	2/17	−	+++	++	−	LT3
14	47	IDC	S-T	I	1.5×1.0	0/4	++	−	+++	+++	134
15	43	IDC	Sci	I	1.5×1.5	0/10	+	+	+	+++	27
16	47	IDC	Sci	II	1.5×1.0	1/12	+	−	+++	−	LT3
17	44	IDC	S-T	II	3.0×3.2	2/13	++	−	+++	+++	11
18	50	IDC	P-T[9]	I	3.9×3.5	5/17	+	+++	+++	+	LT3
19	61	IDC	S-T	III	8.0×7.0	11/25	++++	+++	+++	−	ND
20	89	IDC	Sci	II	3.0×2.7	1/14	+	−	+++	++	ND
21	44	IDC	Sci	I	2.0×2.0	0/11	+	−	++++	+	53
22	44	IDC	Sci	II	4.0×2.0	1/10	±	−	+++	+	ND
23	49	IDC	Sci	II	1.6×1.8	0/11	+	+	+++	+	ND
24	33	IDC	P-T	III	1.7×0.5	1/21	+++	++	+++	−	ND
25	68	IDC	Sci	III	2.0×2.5	4/12	±	−	+++	+++	LT3

[1] Japan Mammary Cancer Society; [2] Histological grade was determined using a modification of Bloom and Richardson's method (Elston et al. 1982); [3] Dextran coated charcoal method (fmol/mg); [4] Invasive ductal carcinoma; [5] Carcinoma with metaplasia squamous type; [6] Mucinous carcinoma; [7] Solid-tubular carcinoma; [8] Scrirrhous carcinoma; [9] Papillotubular carcinoma; [10] Less than 3; [11] Not detected; −: negative; ±: 1%~5% positive; +: 5%~25% positive; ++: 25%~50% positive; +++: 50%~75% positive; ++++: more than 75% positive

Table 3. Details of the antibodies used in this study

Antibody	Source	Dilution	Fixation	Method
c-myc (OM-11-906)	Cambridge research biochemicals	1:100	10% formalin	indirect
EGF-R (RPN513)	Amersham	1:50	100% acetone	indirect
c-erbB-2 (pAb1)	Triton biosciences inc	1:10	10% formalin	indirect
ER	Abbott laboratories kit	1:1	10% phosphate buffered formalin	PAP

Results

The immunoreactivities of oncogene products and ER are shown in Tables 1 and 2. Figure 1 shows demonstrable immunohistochemical staining of c-erbB-2, EGFR, c-myc and ER in breast cancers. C-erbB-2 product (Fig. 1 A) and EGFR (Fig. 1 B) were localized on the cell membrane whereas c-myc protein (Fig. 1 C) and ER (Fig. 1 D) were localized in the nuclei. The incidence of positive staining (+ or more intense) in the fibroadenomas was follows; c-erbB-2 90.9%, EGFR 72.6%, c-myc 81.8%, ER 100%. In the malignant lesions, c-erbB-2 was 80%, EGFR was 40%, c-myc was 96% and ER was 28% positive. Figure 2 shows a staining series of fibroadenoma, case # 1. There was no statistical difference between expression of c-erbB-2 among the fibroadenomas and the malignant breast lesions, but the immunoreactivities of EGFR and ER were more frequently demonstrated in the fibroadenomas (chi square test, $p < 0.05$). In breast cancers, there was reciprocal correlation between the expression of EGFR and ER (chi square test, $p < 0.05$). Regarding the expression of ER, the coincidence of immunohistochemistry and DCC method was as high as 85% in breast cancers. Figure 3 shows a staining series of invasive ductal carcinoma (IDC), case # 19, with lymph node metastasis whereas Fig. 4 demonstrates a case of IDC, case # 15, without lymph node metastasis. Table 4 demonstrates the relationship between immunoreactivity and lymph node metastasis. In 14 cases with lymph node metastasis, the incidence of positive staining was c-erbB-2: 78.6%, EGFR: 42.9%, c-myc: 92.9% and ER: 50%. In comparison, in 11 cases without lymph node metastasis, the incidence of c-erbB-2 was as low as 63.6% (chi square test, $p < 0.05$), whereas the expressions of EGFR (36.6%), c-myc (100%), and ER (50%) were not significantly different. All 7 cases with strong immunoreactivity (+ + + or + + + + +) for c-erbB-2 were accompanied by lymph node metastasis, and 3 of them (42.9%) also showed strong reactivity for EGFR. In 7 cases with the moderate reactivity (+ + or more intense) for EGFR, 6 cases (85.7%) showed lymph node metastasis. Regarding the size of primary tumours, in 13 cases with a tumour greater than 3.0 cm in diameter, the incidence of positive staining was c-erbB-2 69.2%, EGFR 38.5%, c-myc 100% and ER 61.5%. However, in 12 cases with the tumour less than 3.0 cm in diameter, the incidence was 83.3% for c-erbB-2, 38.5% for EGFR, 91.7% for c-myc and 75% for ER. Regarding the size of the primary tumour, there was no statistical significance to the expression of c-erbB-2, EGFR, c-myc or ER. The histological grade is demonstrated in Table 1. There was a statistically significant correlation with the expression of ER but not with expression of c-erbB-2, EGFR, c-myc (chi square test, $p < 0.05$).

Discussion

The immunohistochemical detection of oncogene products in breast cancer and its relationship with a poor prognosis, short post-relapse survival, primary tumour size and histological grade has been described recently using paraffin embedded tissue (Berger et al. 1988; Vijver et al. 1988; Wright et al. 1989). However, there have been no solid agreements on the relationship between c-erbB-2 gene products and nodal metastasis using immunohistochemistry as the method of investigation. The significant correlation between neu gene (c-erbB-2) and lymph node status has been reported to be related to gene amplification by dot blot analysis (Slamon et al. 1987) and to overexpression of oncogene product using immunohistochemistry on paraffin-embedded tissues (Berger et al. 1988). Vijver et al. 1988, demonstrated neu gene product overexpression in stage II breast cancer and suggested that this overexpression might have been related to an early step in development of a distinct histological type of carcinoma of the breast. In contrast, Wright et al. 1989 have studied 185 paraffin-embedded breast cancers and correlated the c-erbB-2 positive staining with negative ER status and high histological grade. However neither group showed any association of c-erbB-2 expression with either nodal metastasis, tumour size or EGFR status. In the present study, we have dem-

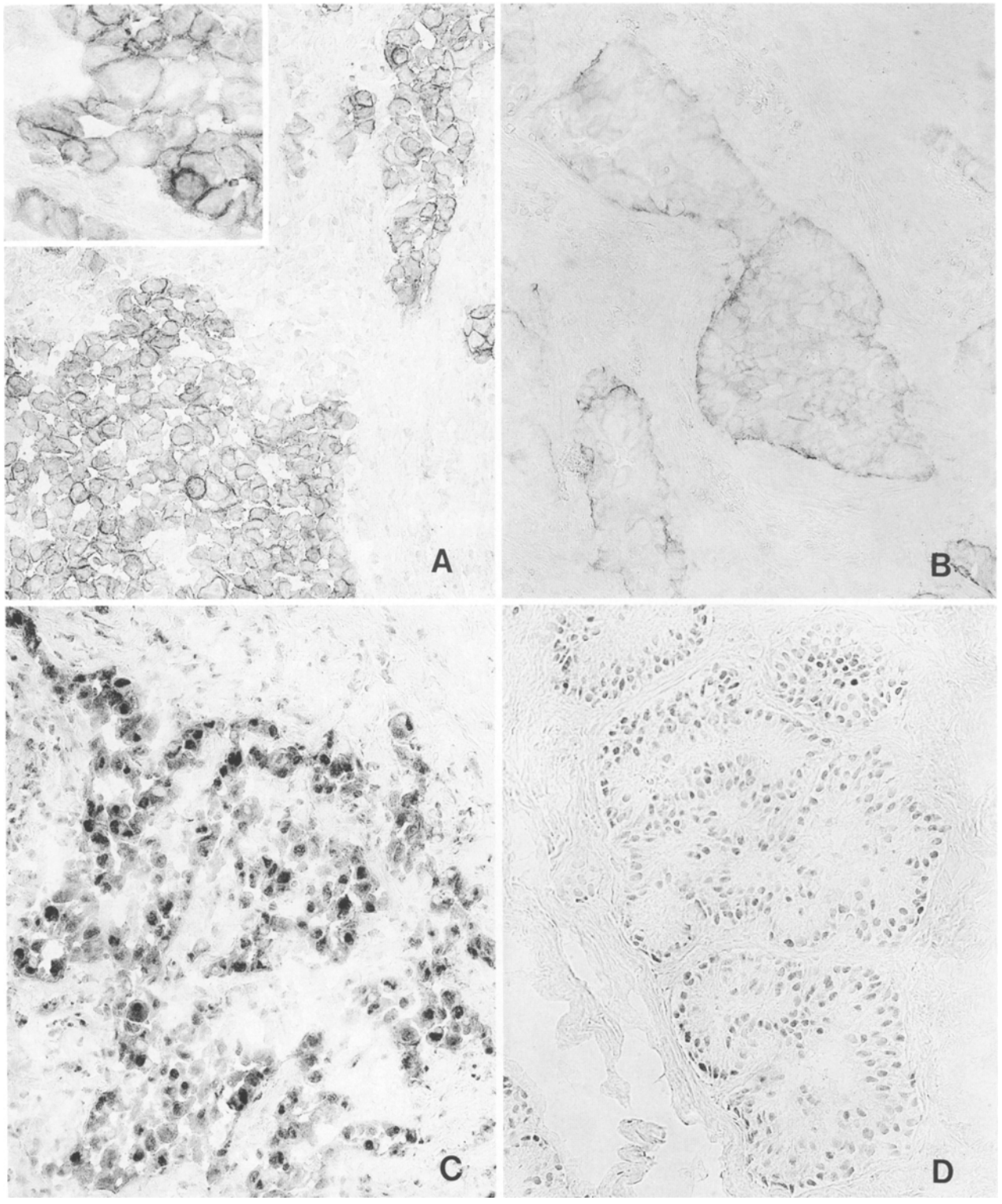

Fig. 1. Representative immunohistochemical staining for oncogene products and estrogen receptor in breast cancer. (**A**) Localization of c-erbB-2 product (×400). (Inset ×800) c-erbB-2 was localized on the cell membrane. (**B**) Localization of EGFR (×400). EGFR was localized on cell membrane. (**C**) Localization of c-myc protein (×400). c-myc was localized in the nuclei. (**D**) Localization of ER (×400). ER was localized in the nuclei

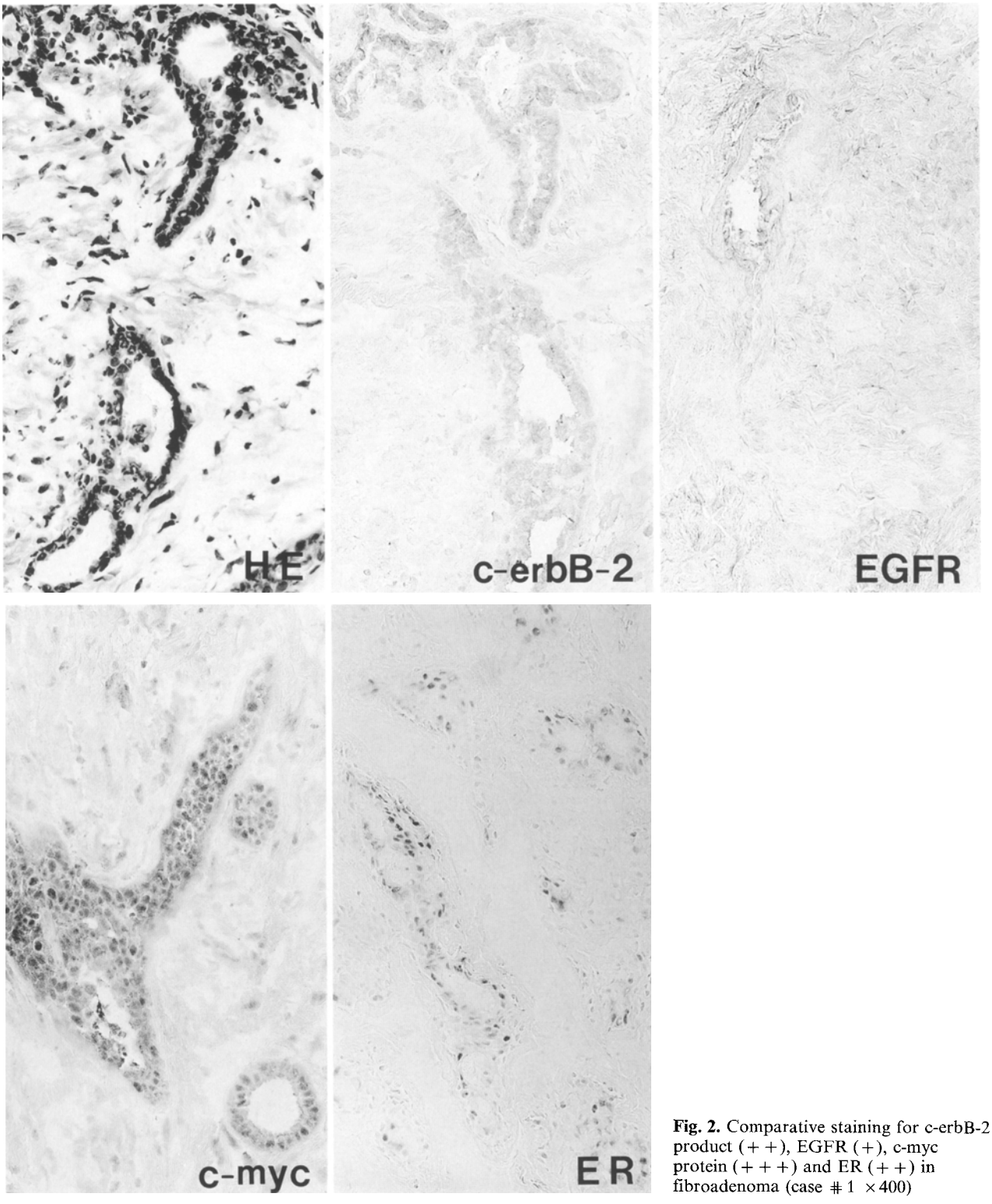

Fig. 2. Comparative staining for c-erbB-2 product (++), EGFR (+), c-myc protein (+++) and ER (++) in fibroadenoma (case # 1 ×400)

onstrated successfully the correlation between expression of c-erbB-2 and lymph node status using fresh frozen tissues. As the function of these oncogenes should be exerted by their products, we believe that the immunohistochemical expression of the oncogene products are very important in prognosis.

The immunohistochemical expression of oncogene products and ER were not only recognized in malignant lesions but also in benign lesions of

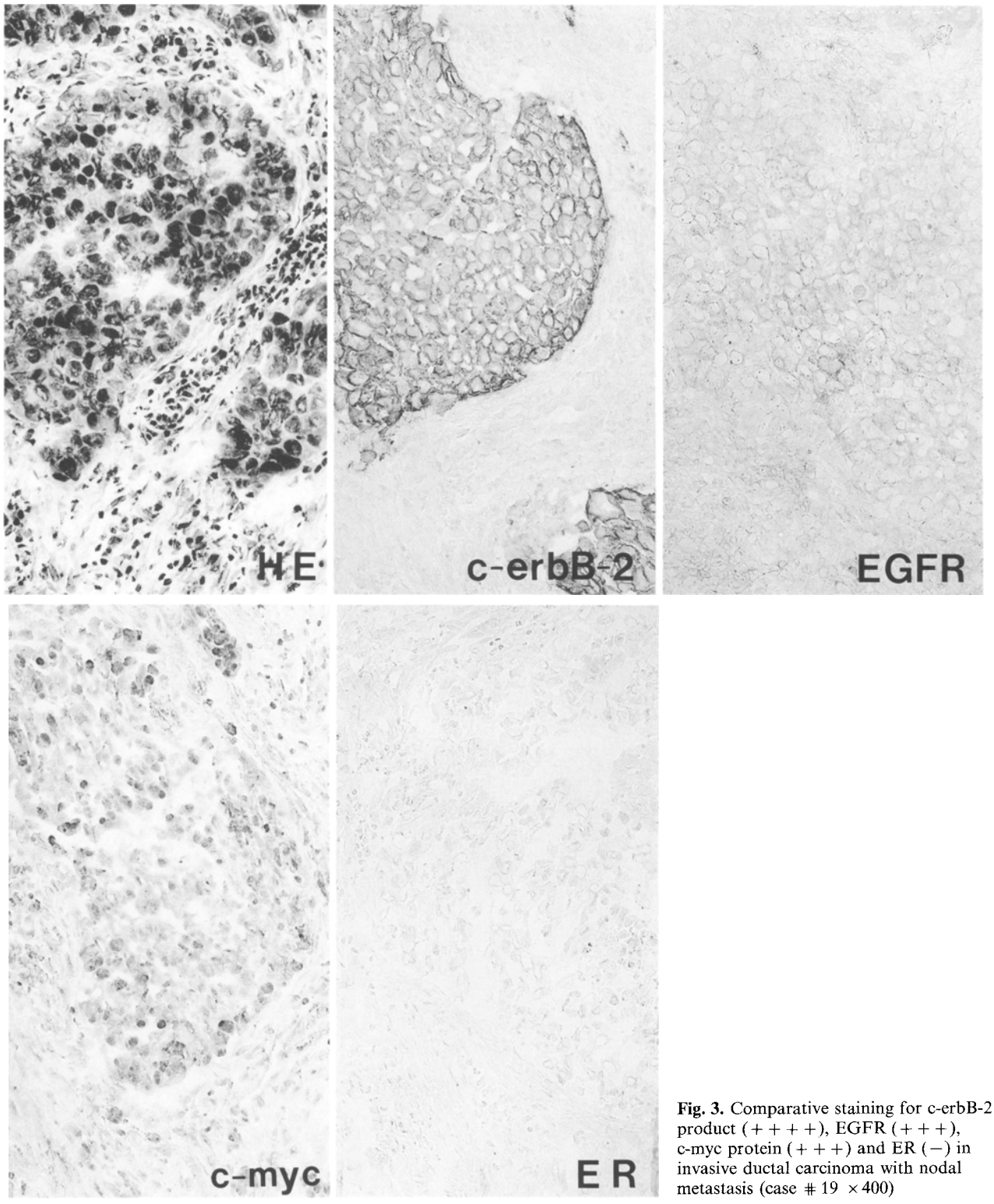

Fig. 3. Comparative staining for c-erbB-2 product (+ + + +), EGFR (+ + +), c-myc protein (+ + +) and ER (−) in invasive ductal carcinoma with nodal metastasis (case # 19 ×400)

the breast. There was no statistical significance in c-erbB-2 and c-myc expression between fibroadenomas and breast cancers, but the immunoreactivity of EGFR and ER was more frequently seen in the former. In fibroadenomas, the expression of c-erbB-2 and c-myc may be related to their growth activity in a benign fashion.

The c-erbB-2 proto-oncogene amplification or oncogene product overexpression was reported from 14% to 58% in human breast cancer by dot

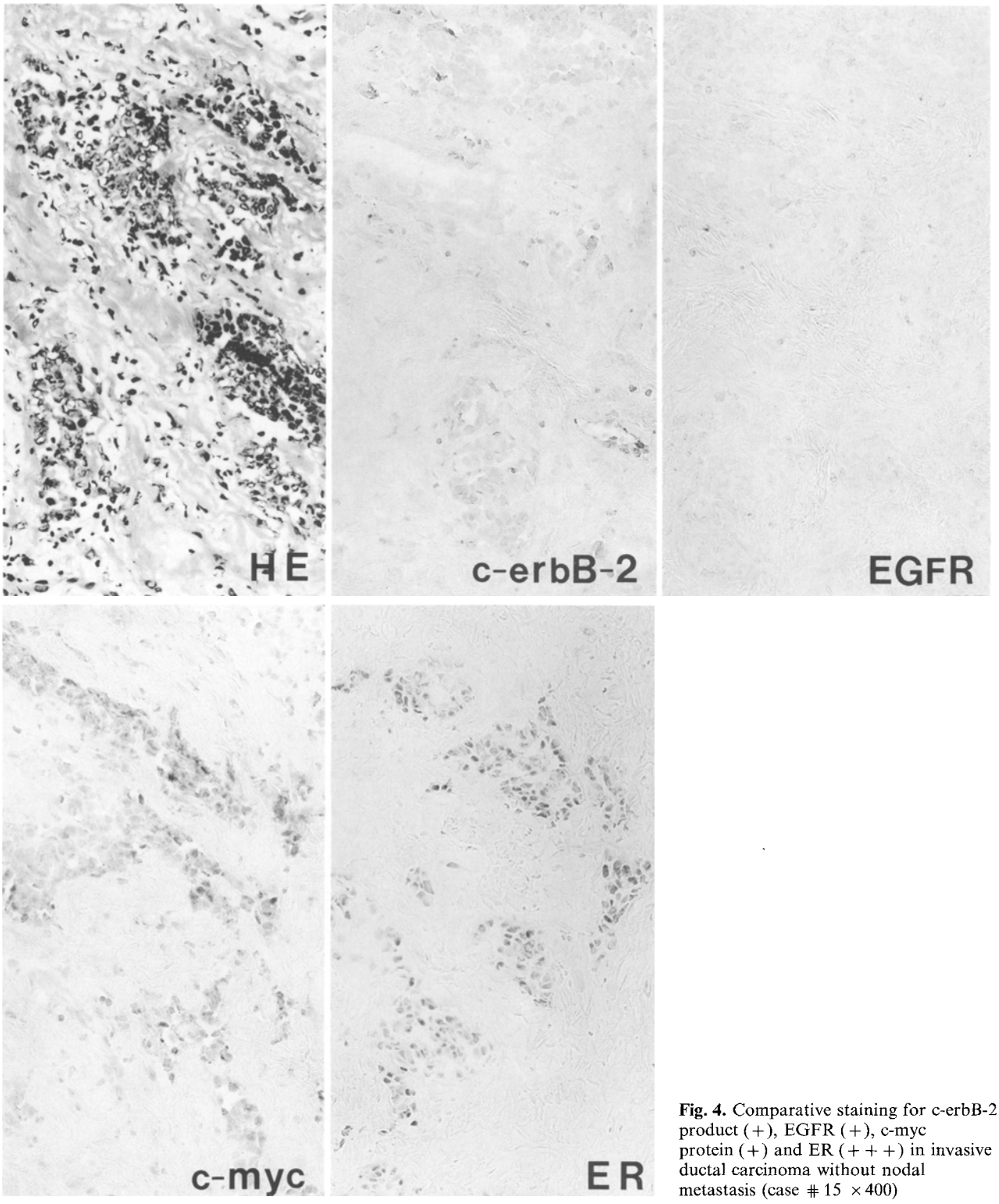

Fig. 4. Comparative staining for c-erbB-2 product (+), EGFR (+), c-myc protein (+) and ER (+ + +) in invasive ductal carcinoma without nodal metastasis (case # 15 ×400)

blot analysis or immunohistochemistry (King et al. 1985; Yokota et al. 1986; Mori et al. 1987; Slamon et al. 1987; Varley et al. 1987; Venter et al. 1987; Vijver et al. 1987; Zhou et al. 1987; Berger et al. 1988; Vijver et al. 1988; Wright et al. 1989). In our study, positive immunoreactivity for c-erbB-2 in breast cancers was as high as 80%. Such our higher incidence when compared with other reports is considered to have resulted from using fresh frozen tissues to improve the preservation

Table 4. Immunohistochemical staining and lymph node metastasis

Case	c-erbB-2	EGFR	c-myc	ER	ER (DCC)	LN meta
1	++	−	+++	+++	40	0/ 5
2	+++	−	+++	−	LT3	2/20
3	−	−	+++	−	LT3	0/22
4	±	−	++++	+++	176	0/16
5	+++	+++	±	−	ND	1/21
6	+	−	+++	+++	231	0/18
7	+	+	+	+++	95	0/22
8	+++	+++	+++	−	LT3	44/48
9	++++	−	+++	++	50	10/17
10	+++	−	+++	+++	LT3	1/14
11	±	+++	+	++	38	0/40
12	+	−	+++	++	50	0/25
13	−	+++	++	−	LT3	2/17
14	++	−	+++	+++	134	0/ 4
15	+	+	+	+++	27	0/10
16	+	−	+++	−	LT3	1/12
17	++	−	+++	+++	11	2/13
18	+	+++	+++	+	LT3	5/17
19	++++	+++	+++	−	ND	11/25
20	+	−	+++	++	ND	1/14
21	+	−	++++	+	53	0/11
22	±	−	+++	+	ND	1/10
23	+	+	+++	+	ND	0/11
24	+++	++	+++	−	ND	1/21
25	±	−	+++	+++	LT3	4/12

of the membrane associated antigens comparing to paraffin sections. However, the incidence of strong immunoreactivity (+++ to ++++) of c-erbB-2 was 7/25 (28.0%) which was similar to other reported incidences.

The c-erbB-2 receptor encodes a receptor-like protein which is very similar to, but distinct from, EGFR, and their homology is limited to the region encoding the tyrosine kinase domain (Coussens et al. 1985; Semba et al. 1985; Akiyama et al. 1986; Bargmann et al. 1986; Yamamoto et al. 1986; Yokota et al. 1986). In 1987, Sainsbury et al. demonstrated a significant association between tumour size and EGFR by receptor assay in 135 primary breast cancers. Such the association could not be shown by immunohistochemistry in our series, nevertheless, the reciprocal correlation between the expression of EGFR and ER was similar to their results.

The c-myc oncogene or protein shows a high degree of evolutionary conservation (Persson et al. 1984), occurs at particular stages of the cell cycle (Rabbitts et al. 1985), in particular stages of differentiation (Gowda et al. 1986) or after mutation (Zimmerman et al. 1986; Pfeifer-Ohlsson et al. 1984). We demonstrated the expression of c-myc protein on the nuclei in many cases, and there was no significant correlation of its immunoreactivity between benign and malignant breast lesions. No correlation regarding either the lymph node metastasis or primary tumour size protein was seen with c-myc.

Regarding the immunoreactivity of c-erbB-2 to lymph node status, its strong immunoreactivity (+++ to ++++) was detected in 7 cases (50%) with lymph node metastasis but not observed in cases without nodal metastasis. The difference of c-erbB-2 immunoexpression between the cases with lymph node metastasis and the cases without nodal metastasis was statistically significant and suggested its overexpression to be more likely to be associated with nodal metastasis. Interestingly, the strong reactivity of c-erbB-2 did not correlate with the histological grade but could be detected in 3 cases containing a tumour less than 2.0 cm with positive nodal metastasis.

Surgical treatment of breast cancer is changing from aggressive Halsted, Patey or Auchincloss method to limited lumpectomy, quadrantectomy or simple mastectomy. It is important for surgeons to be able to predict, preoperatively, the prognosis especially with regard to the situation of lymph node metastases, in order to determine the proper manipulation and necessity for adjuvant therapy. In the needle aspirates or biopsy specimens, in addition to the assessment of histological grading and the status of ER, it is expected that demonstration of expression of c-erbB-2 by immunohistochemistry may provide preoperative prediction of the biological behavior of the carcinoma.

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