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Determination of c-erbB-2 Protein in Primary Breast Cancer Tissue Extract Using an Enzyme Immunoassay

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The c-erbB-2 protein in breast cancer tissue extract was determined by using an enzyme-immunoassay (EIA) to see whether the quantitative determination of the oncoprotein correlates with the results of immunohistochemistry and other prognostic factors. Primary breast cancer from 104 patients was assayed for c-erbB-2 protein with an EIA that used two monoclonal antibodies directed against the extracellular domain of the protein. Pelleted tissue homogenate prepared routinely for hormone receptor assay was used as the starting material. The mean quantity of c-erbB-2 protein was 695 unit/mg protein (range 23 to 5939), and this correlated well with the results of immunohistochemical staining ($P<0.00001$). It was found that 17.3%(18/104) of all tumors contained amounts of c-erbB-2 protein exceeding 1000 units/mg protein. All tumors with negative or weakly positive staining contained the oncoprotein as less than 1000 units/mg protein. The content of c-erbB-2 protein was correlated with the histologic grade ($P=0.0022$), mitotic index ($P=0.0002$) and degree of nuclear atypia ($P=0.013$). It was inversely correlated with progesterone receptor ($P=0.006$) and less strongly with estrogen receptor status ($P=0.016$). Values of hormone receptor concentration and c-erbB-2 protein content showed a hyperbolic relationship that suggested biological interactions between c-erbB-2 protein and steroid hormone receptors. We conclude that c-erbB-2 protein in tissue extracts of primary breast cancer can be determined reliably by EIA, and it seems feasible to explore further the advantages of introducing EIA as a routine laboratory examination for providing additional information about the biological aspects of breast cancer.

Key words: Breast neoplasm — Oncogene — c-erbB-2 — Growth factor —Hormone receptor

Since Slamon *et al.* first reported the prognostic significance of c-*erb*B-2 oncogene amplification in patients with primary breast cancer, many attempts have been made to examine the usefulness of oncoprotein as a predictive factor of outcome for breast cancer patients after surgery.[1-3] Although the prognostic significance of c-*erb*B-2 has been demonstrated in certain subsets of primary breast cancer patients, no conclusion has been reached concerning the predictive value of the oncogene in node-negative patients with common infiltrating ductal carcinoma, the subgroup for which definition of a better prognostic index is most urgently required.[4-9] It was stated in a recent editorial that there is little justification for the introduction of c-*erb*B-2 oncogene measurement into routine clinical practice because it has still not been confirmed to provide any predictive information additional to that obtained by more traditional tests.[10] However, some recent studies have attempted to address the relationship between c-*erb*B-2 measurement and other clinically relevant aspects of breast cancer such as resistance to chemotherapeutic and hormonal agents, and the tendency of the cancer to metastasize to certain organ systems.[9, 11, 12]

Genomic DNA amplification of the c-*erb*B-2 oncogene is the most common mechanism of activation of the gene, leading to overexpression of c-erbB-2 protein. Another mechanism of c-*erb*B-2 activation is overexpression of c-erbB-2 protein without gene amplification.[3] Therefore, detection of c-erbB-2 protein overexpression is thought to be a better indicator of c-erbB-2 activation than detection of DNA amplification.[13, 14] The most accurate methods so far for detecting c-erbB-2 protein overexpression are the Western blot technique and immunoprecipitation.[15] However, both of these methods require specialized laboratory techniques and are not suited for practical laboratory use.[3] Determination of protein expression with immunohistochemical staining using paraffin-embedded archival material is a commonly employed technique for revealing c-erbB-2 abnormalities. The positivity of immunohistochemical staining for the oncoprotein ranges from 15% to 25% in primary breast cancer.[16, 17] Although the technique is reliable, there is inconsistency in the criteria for assessing the results of staining, and variations in the methods of tissue fixation

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have produced conflicting results among studies.[3] Recently, an enzyme immunoassay (EIA) was developed using two monoclonal antibodies directed against the extracellular domain of the c-erbB-2 protein, and it revealed that levels of the protein in the serum and malignant effusion of breast cancer patients were elevated.[18] If the content of c-erbB-2 protein in tumor tissue could be assessed reliably with this EIA, it would provide another technique of c-erbB-2 measurement with advantages over immunohistochemical staining, being rapid and allowing easy control of the assay procedure quality. In this report, we describe the use of this EIA to quantify c-erbB-2 protein in extracts of primary breast cancer tissues, all of which were also stained immunohistochemically for the oncoprotein. The results obtained with the two techniques were then compared in order to assess the utility of the EIA. Quantitative assessment of c-erbB-2 content was also performed in relation to other prognostic factors of primary breast cancer.

PATIENTS AND METHODS

Patients Tumor tissues from 104 patients diagnosed as having primary breast cancer were collected between October 1991 and May 1992. The characteristics of the patients are shown in Table I. All patients underwent either radical or modified radical mastectomy at the National Cancer Center Hospital. These patients had received no chemotherapy, endocrine therapy or radiation therapy prior to surgery. Fibroadenoma tissues from ten patients were also collected during the same period. Samples were sent immediately after surgical resection to the Pathology Division and divided into two pieces. One piece was used for pathological examination and the other was frozen in liquid nitrogen and sent to the Laboratory of Endocrinology for determination of steroid hormone receptor content.

Tissue preparation Approximately 0.4 g of tumor tissue was pulverized in a freezer-mill (Spex Industries, Inc., Metuchen, NJ) and homogenized in 3 ml of buffer solution (0.01 mol/liter Tris, 0.001 mol/liter EDTA, 0.012 mol/liter thioglycerol, 10% glycerol, pH 7.4) with a Polytron homogenizer (Kinematica GmbH, Lucerne, Switzerland). The homogenate was centrifuged at 105,000*g* at 4°C for 60 min and the supernatant fraction was assayed for estrogen receptor (ER) and progesterone receptor (PgR) using EIA kits (Abbot Laboratories, North Chicago, IL).[19] The pelleted material was stored at −70°C. All patients with unilateral disease whose tissue pellets had been stored under ideal conditions were included in this study. The number of patients with breast cancer in this study was 55% of all patients who underwent breast surgery at the hospital over the eight-month period.

Table I. Characteristics of the Patients

Number of patients		104[a]
Menopausal status	pre	52
	post	52
Clinical stage[b]	I	34
	IIA	42
	IIB	17
	IIIA	6
	IIIB	4
	IV	1
Pathologic tumor size (cm)	≤2	49
	2–5	48
	>5	7
Positive nodes	0	56
	1–3	30
	4–9	9
	≥10	9
Hormone receptor status (ER/PgR)[c]	+/+	71
	+/−	19
	−/+	3
	−/−	11

a) Mean age of the patients was 53.9 (range: 38–83).
b) Clinical stage according to the American Joint Committee on Cancer.
c) Cut-off points: ER, 13 fmol/mg protein; PgR, 10 fmol/mg protein.

EIA for c-erbB-2 protein The pelleted tumor tissue homogenate stored at −70°C was resuspended in extraction solution (0.01 mol/liter sodium phosphate, 0.15 mol/liter sodium chloride, 1% Triton X-100, 0.5% sodium deoxycholate, 1% sodium dodecyl sulfate, and 0.001 mol/liter phenylmethylsulfonyl fluoride, pH 7.2). Determination of protein in the cytosol and the pellet extract was performed by the Coomassie brilliant blue method (Bio-rad Laboratories, Richmond, CA). c-erbB-2 protein in the tissue extract was assayed with an enzyme immunoassay kit, following the protocol recommended by the manufacturers (Triton Diagnostics, Alameda, CA). The monoclonal antibodies used in this assay (TAb 259 and TAb 257) are reactive with the extracellular domain of c-erbB-2 protein.[20] The assay's sensitivity and specificity have been reported previously.[18]

Immunohistochemistry The antibody used for immunohistochemical staining of c-erbB-2 protein was raised in a rabbit after immunizing with a synthetic peptide corresponding to a portion of the cytoplasmic domain of the c-*erb*B-2 oncogene product (amino acid residues 1242 to 1255).[21] The staining procedure has been described in detail previously.[16] The staining intensity was scored using a scale of negative/weakly positive/strongly positive, as shown with photomicrographs in the previous

report. Staining was regarded as strongly positive when an intense reaction was observed in more than 25% of tumor cells. The results of staining were always checked by the same individual (H.T.).

Clinical data All clinical data were retrieved from the medical charts of the patients. Each pathological report included the size of the tumor, number of lymph node metastases and histologic grade, which was scored according to a modification of the grading system of Bloom and Richardson.[22]

Statistical analysis Comparison of the means of extracted c-erbB-2 content in two and more than two categories of other prognostic factors were performed by using Student's two-tailed unpaired t test and one-way analysis of variance followed by Duncan's multiple range test, respectively. Statistical calculations were performed with a statistical package, SPSS (SPSS Japan Inc., Tokyo) on an Apple personal computer, Macintosh (Cupertino, CA).

RESULTS

c-erbB-2 protein content in tumor extract The content of extractable c-erbB-2 protein in the pellets of breast cancer and fibroadenoma tissue ranged from 23 to 5939 unit/mg protein (mean; 695) and from 188 to 920 (mean; 244), respectively. The contents of c-erbB-2 protein in breast cancer with negative (n=28), weakly positive (n=53) and strongly positive staining (n=23) for the protein are illustrated in Fig. 1. The content was significantly higher in breast cancer tissues with strongly positive staining than in tissues belonging to the other two categories ($P<0.00001$). The contents of extractable c-erbB-2 protein did not differ among fibroadenoma and breast cancer tissues with negative or weak staining, and all tissues in these groups contained less than 1000 units of c-erbB-2 protein/mg tissue protein. Among the 23 specimens that showed strongly positive staining, five contained less than 1000 units of oncoprotein/mg protein. When only strongly positive staining was considered positive, a cut-off point of 1000 unit/mg protein of extracted c-erbB-2 protein in the tissue pellet effectively distinguished samples with positive staining from those with negative staining (sensitivity; 78%, specificity; 100%).

Relationship between c-erbB-2 protein content and other prognostic factors c-erbB-2 protein content was significantly high in breast cancer with higher histological grade ($P=0.0022$), mitotic index ($P=0.0002$) and nuclear atypia ($P=0.013$) but no correlation was observed between the oncoprotein content and the degree of structural atypia (Table II). When the c-erbB-2 protein content was compared on the basis of axillary lymph nodal status and the clinical stage, there was no difference in content among the groups. There was a tendency for larger tumors to contain higher amounts of the oncoprotein. The content of c-erbB-2 protein was significantly high in tumors that were negative for ER and PgR ($P=0.016$ and 0.006, respectively). This association of c-

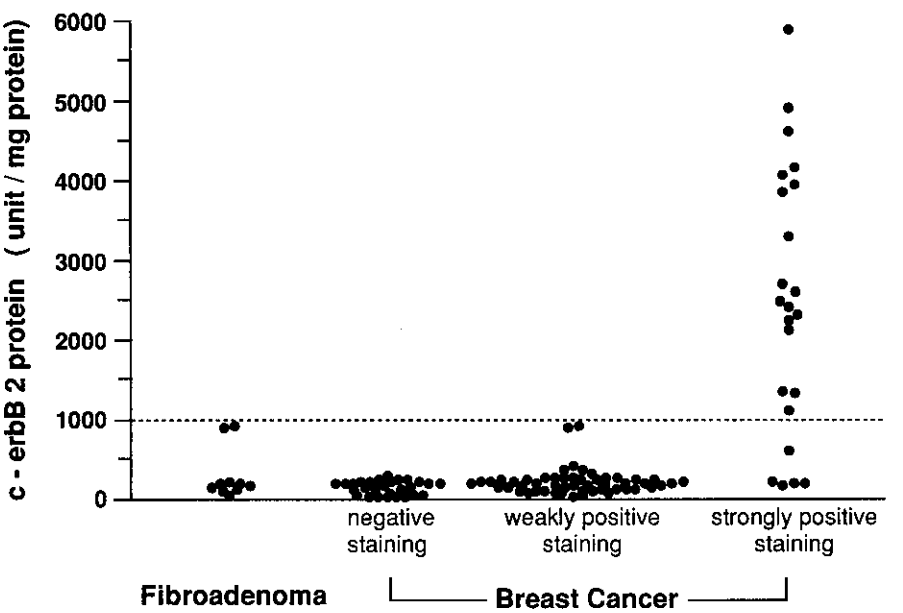

Fig. 1. c-erbB-2 protein content in tissue extract of 10 fibroadenomas and 104 breast cancer tissues. The breast cancer tissues are categorized according to the results of immunohistochemical staining.

Table II. Relationships between c-erbB-2 Protein Content and Other Prognostic Factors

Factor	Patient group	Number of patients	c-erbB-2 content (U/mg protein)[a]	P value
Pathological tumor size (cm)	≤2	49	476±126	0.08[b]
	>2	55	890±198	
Positive nodes	0	56	832±185	0.32[c]
	1–3	30	410±150	
	≥4	18	742±309	
Clinical stage	I	34	548±182	0.54[c]
	II	59	718±164	
	III, IV	11	1021±494	
Nuclear atypia	1	8	104±28	0.013[c]
	2	47	416±145	
	3	49	1058±206[d]	
Architectural atypia	1	10	573±377	0.14[c]
	2	37	340±127	
	3	57	907±192	
Mitotic figures	1	28	186±17	0.0002[c]
	2	31	349±100	
	3	45	1250±250[d]	
Histologic grade	1	8	169±25	0.0022[c]
	2	47	327±96	
	3	49	1133±226[d]	
ER	negative	14	1975±529	0.016[b]
	positive	90	496±100	
PgR	negative	30	1305±269	0.006[b]
	positive	74	447±121	

a) Values are mean±standard error.
b) *P* value derived from Student's *t* test.
c) *P* value derived from one-way analysis of variance.
d) Significantly different from the other two values by Duncan's multiple range test ($P=0.05$).

erbB-2 protein content and hormone receptor status was further analyzed. When the determined values of ER and PgR concentration were plotted against c-erbB-2 protein content, a hyperbolic relationship was observed for both ER and PgR (Fig. 2). Tumors with high contents of steroid hormone receptors contained small amounts of c-erbB-2 protein, and tumors with high contents of c-erbB-2 protein had low hormone receptor levels. The relationship was more obvious when the oncoprotein content was compared with PgR than with ER.

DISCUSSION

The present data showed that the content of c-erbB-2 protein in tissue pellets of primary breast cancer determined by EIA correlated well with the results of immunohistochemical staining for the oncoprotein. When a cut-off point was set tentatively at 1000 units of extracted oncoprotein/mg tissue protein, 17.3% of the primary breast cancers were considered positive. Five tumors among the 23 which showed strongly positive staining for the c-erbB-2 protein contained small amounts of extractable oncoprotein, and were considered to be negative for oncoprotein expression based on the above cut-off point. We were unable to identify any clinical or pathological characteristics common to these five exceptions. It might be argued that the discrepancy was due to the difference in the epitopes recognized by the two methods for detecting the expression of the protein. The monoclonal antibodies used in the EIA recognize the extracellular domain of the oncoprotein, whereas the immunohistochemical staining employed a polyclonal antibody which was raised by immunizing a rabbit with a synthetic peptide corresponding to a portion of the cytoplasmic domain. It has been demonstrated that a truncated form of epidermal growth facter (EGF) with impaired binding capacity towards EGF is encoded by c-erbB genes with deletion mutations within the extracellular ligand-binding domain of the EGF receptor.[23] If this is the case for c-erbB-2, it could explain the discrepancy observed in

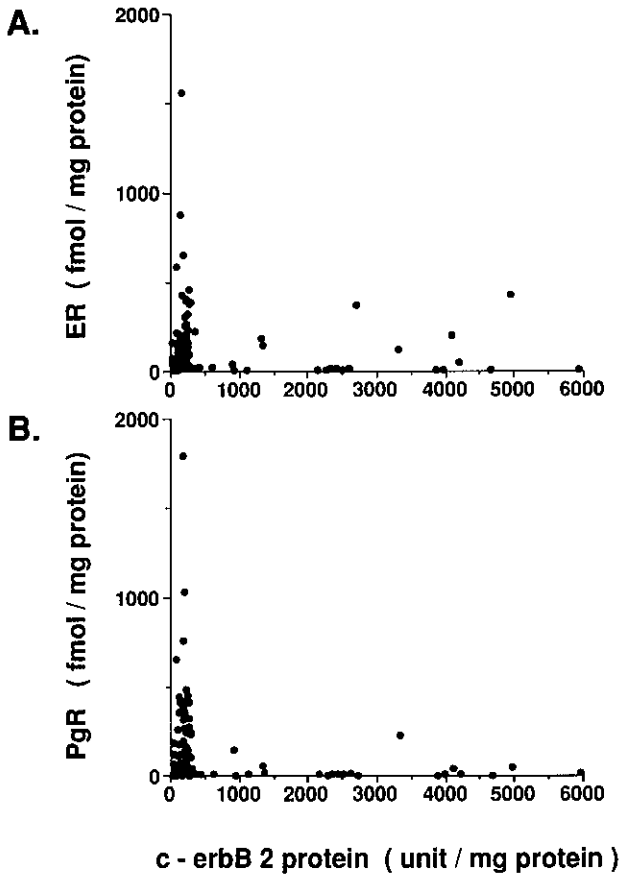

Fig. 2. Relationship between (A) ER and (B) PgR values and c-erbB-2 protein content in 104 breast cancer tissues. The hormone receptors and the oncoprotein content showed a hyperbolic relationship. This is more obvious for PgR than for ER.

the five cases. A cell-associated cleavage product made up of the transmembrane and cytoplasmic domains remaining in the cells after proteolytic release of the extracellular domain of the c-erbB-2 product could also account for the discrepancy.[24] A further study with use of the same antibody against the same epitope in EIA and immunohistochemistry would allow a more accurate conclusion. Another possible explanation for the inconsistency is heterogeneous expression of c-erbB-2 protein within a single tumor. This may occur when a tumor contains both intraductal and infiltrating components, the former showing intense staining.[25,26] Because the c-erbB-2 protein content determined by EIA represents an average of that in the tumor as a whole, a high content of c-erbB-2 protein in a portion showing intense staining may be diluted by the negatively stained area after tumor homogenization. Whatever the reason for this inconsistency, we cannot yet reach a definite conclusion about the superiority of either of these methods for predicting the prognosis of breast cancer patients before the survival data of the patient population are obtained.

The relationship between expression or amplification of c-erbB-2 and other prognostic factors has been extensively reported. In this study, a strong correlation was observed between c-erbB-2 protein content and histologic grade, particularly the mitotic index among the three components of the grading system. This observation is in accord with previous reports.[9,14,17,22] Our study found no association between c-erbB-2 protein content and axillary lymph nodal status. There seems to be little agreement in the literature concerning this association. Because axillary nodal status is the most reliable prognostic indicator of primary breast cancer patients and most reports have indicated that c-*erb*B-2 overexpression or amplification is a prognostic factor independent of nodal status, some investigators have hypothesized that the two factors represent two different aspects of the pathogenesis of breast cancer spread, that is, nodal status reflects relative chronological age and c-*erb*-2 expression is an indicator of cellular kinetics of breast cancer.[2,27,28] Our finding that c-erbB-2 protein content was well associated with the mototic index, which is a morphological indicator of cell kinetics, supports this view.

We observed a strong association between c-erbB-2 protein content and hormone receptor status, especially PgR. In the literature, the association of c-*erb*B-2 oncogene expression or amplification with steroid receptor status is controversial.[1,5,6,9,14,15,29–33] In almost all published studies, the hormone receptor status of patients was determined by the conventional dextran-coated charcoal method, which presents intrinsic difficulties with quality control and shows relatively large interinstitutional variability; furthermore, the results are reported simply as either positive or negative. The present study is the first to show a quantitative relationship between hormone receptor levels and the content of c-erbB-2 protein. The hyperbolic relationship between the determined values of ER or PgR concentration and the content of c-erbB-2 protein indicates there must be some biological interplay between these two parameters of breast cancer. Such a relationship is supported by several *in vitro* studies, where estradiol dose-dependently inhibited c-erbB-2 mRNA and protein in ER-positive breast cancer cell lines.[34–36] Although the nature of this relationship is still unknown, quantitative assessment of c-erbB-2 protein by EIA provided information additional to that obtained by qualitative assessment methods such as immunohistochemical staining with regard to involvement of the two signal transduction pathways in growth regulation of breast cancer cells, one being related to the c-erbB-2 protein and the other to sex steroid hormone

receptors. The clinical observation that c-erbB-2 protein overexpression is associated with a lack of response to endocrine therapy on relapse can be fully explained by the present results.[11] Furthermore, it is speculated that ER-positive breast cancer with positive c-erbB-2 may be equipped with an impaired post ER signal transduction pathway and be less responsive to endocrine therapies than breast cancer with positive ER and negative c-erbB-2, based on the fact that stimulation of ER-positive cells results in down-regulation of c-erbB-2 expression.[35]

Even though the value of c-erbB-2 as a prognostic indicator in patients with primary breast cancer has been studied extensively by many clinical investigators, it is claimed that introduction of c-*erb*B-2 oncogene measurement into clinical practice is not justified.[10] We cannot address directly the prognostic utility of the oncoprotein on the basis of the present data because our patient cohort was selected from a group who underwent surgery very recently. However, we did confirm that c-erbB-2 oncoprotein content determined by EIA using material that would otherwise have been discarded could provide information on the expression of the oncoprotein equivalent to that obtained by immunohistochemistry. Standardization of tissue fixation methods and establishment of minimum criteria for interpreting the results of the c-erbB-2 protein staining are mandatory before immunohistochemical staining can provide valid and concordant results between institutes. On the other hand, EIA is a very simple and rapid procedure which can be easily standardized according to the usual quality control methodology used in laboratory examinations. On the basis of the present findings, it seems justified to explore further the advantages of introducing EIA as a routine laboratory test for providing additional information about the biological aspects of breast cancer, the prognostic significance of c-*erb*B-2 oncogene activation, and the possible utility of new treatment approaches.[37]

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REFERENCES

1) Slamon, D. J., Clark, G. M., Wong, S. G., Levin, W. J., Ullrich, A. and McGuire, W. L. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/*neu* oncogene. *Science,* **235,** 177–182 (1987).
2) McGuire, W. L., Tandon, A. K., Allred, D. C., Chamness, G. C., Ravdin, P. M. and Clark, G. M. Treatment decisions in axillary node-negative breast cancer patients. *J. Natl. Cancer Inst. Monogr.,* **11,** 173–180 (1992).
3) Singleton, T. P. and Strickler, J. G. Clinical and pathologic significance of the c-erbB-2 (HER-2/*neu*) oncogene. *Pathol. Annu.,* 165–190 (1992).
4) van de Vijver, M. J., Peterse, J. L., Mooi, W. J., Wisman, P., Lomans, J., Dalesio, O. and Nusse, R. *Neu*-protein overexpression in breast cancer. Association with comedo-type ductal carcinoma in situ and limited prognostic value in stage II breast cancer. *N. Engl. J. Med.,* **319,** 1239–1245 (1988).
5) Clark, G. M. and McGuire, W. L. Follow-up study of HER-2/*neu* amplification in primary breast cancer. *Cancer Res.,* **51,** 944–948 (1991).
6) Berns, E. M. J. J., Klijn, J. G. M., van Putten, W. L. J., van Staveren, I. L., Portengen, H. and Foekens, J. A. c-*myc* amplification is a better prognostic facter than *HER2/neu* amplification in primary breast cancer. *Cancer Res.,* **52,** 1107–1113 (1992).
7) Allred, D. C., Clark, G. M., Tandon, A. K., Molina, R., Tormey, D. C., Osborne, C. K., Gilchrist, K. W., Mansour, E. G., Abeloff, M., Eudey, L. and McGuire, W. L. HER-2/*neu* in node-negative breast cancer: prognostic significance of overexpression influenced by the presence of in situ carcinoma. *J. Clin. Oncol.,* **10,** 599–605 (1992).
8) Neville, A. M., Bettelheim, R., Gelber, R. D., Save, S. J., Davis, B. W., Reed, R., Torhorst, J., Golouh, R., Peterson, H. F., Price, K. N., Isley, M., Rudenstam, C.-M., Collins, J., Castiglione, M., Senn, H.-J. and Goldhirsch, A. for the International (Ludwig) Breast Cancer Study Group. Factors predicting treatment responsiveness and prognosis in node-negative breast cancer. *J. Clin. Oncol.,* **10,** 696–705 (1992).
9) Gusterson, B. A., Gelber, R. D., Goldhirsch, A., Price, K. N., Save, S. J., Anbazhagan, R., Styles, J., Rudenstam, C. M., Golouh, R., Reed, R., Martinez-Tello, F., Tiltman, A., Torhorst, J., Grigolato, P., Bettelheim, R., Neville, A. M., Burki, K., Castiglione, M., Collins, J., Lindtner, J. and Senn, H.-J. Prognostic importance of *c-erb*B-2 expression in breast cancer. *J. Clin. Oncol.,* **10,** 1049–1056 (1992).
10) Levine, M. N. and Andrulis, I. The HER-2/*neu* oncogene in breast cancer: so what is new? *J. Clin. Oncol.,* **10,** 1034–1036 (1992).
11) Wright, C., Nicholson, S., Angus, B., Sainsbury, J. R., Farndon, J., Cairns, J., Harris, A. L. and Horne, C. H.

Relationship between c-erbB-2 protein product expression and response to endocrine therapy in advanced breast cancer. *Br. J. Cancer,* **65,** 118–121 (1992).
12) Muss, H., Thor, A., Kute, T., Liu, E., Koerner, F., Berry, D., Cirrincione, C., Budman, D., Wood, W., Barcos, M. and Henderson, I. C. erbB-2 (c-erbB-2; HER-2/neu) and S-phase fraction predict response to adjuvant chemotherapy in patients with node-positive breast cancer: cancer and acute leukemia group B trial 8869. *Proc. Am. Soc. Clin. Oncol.,* **12,** 72 (1993).
13) Iglehart, J. D., Kraus, M. H., Langton, B. C., Huper, G., Kerns, B. J. and Marks, J. R. Increased *erb*B-2 gene copies and expression in multiple stages of breast cancer. *Cancer Res.,* **50,** 6701–6707 (1990).
14) Berger, M. S., Locher, G. W., Saurer, S., Gullick, W. J., Waterfield, M. D., Groner, B. and Hynes, N. E. Correlation of c-erbB-2 gene amplification and protein expression in human breast carcinoma with nodal status and nuclear grading. *Cancer Res.,* **48,** 1238–1243 (1988).
15) Tandon, A. K., Clark, G. M., Chamness, G. C., Ullrich, A. and MuGuire, W. L. HER-2/*neu* oncogene protein and prognosis in breast cancer. *J. Clin. Oncol.,* **7,** 1120–1128 (1989).
16) Tsuda, H., Hirohashi, S., Shimosato, Y., Tanaka, Y., Hirota, T., Tsugane, S., Shiraishi, M., Toyoshima, K., Yamamoto, T., Terada, M. and Sugimura, T. Immunohistochemical study on overexpression of c-*erb*B-2 protein in human breast cancer: its correlation with gene amplification and long-term survival of patients. *Jpn. J. Cancer Res.,* **81,** 327–332 (1990).
17) Toikkanen, S., Helin, H., Isola, J. and Joensuu, H. Prognostic significance of HER-2 oncoprotein expression in breast cancer: a 30-year follow-up. *J. Clin. Oncol.,* **10,** 1044–1048 (1992).
18) Leitzel, K., Teramoto, Y., Sampson, E., Mauceri, J., Langton, B. C., Demers, L., Podczaski, E., Harvey, H., Shambaugh, S., Volas, G., Weaver, S. and Lipton, A. Elevated soluble c-erbB-2 antigen levels in the serum and effusions of a proportion of breast cancer patients. *J. Clin. Oncol.,* **10,** 1436–1443 (1992).
19) Watanabe, T., Wu, J. Z., Morikawa, K., Fuchigami, M., Kuranami, M., Adachi, I., Yamaguchi, K. and Abe, K. *In vitro* sensitivity test of breast cancer cells to hormonal agents in a radionucleotide-incorporation assay. *Jpn. J. Cancer Res.,* **81,** 536–543 (1990).
20) Langton, B. C., Crenshaw, M. C., Chao, L. A., Stuart, S. G., Akita, R. W. and Jackson, J. E. An antigen immunologically related to the external domain of gp185 is shed from nude mouse tumors overexpressing the *c-erb*B-2 (HER-2/*neu*) oncogene. *Cancer Res.,* **51,** 2593–2598 (1991).
21) Akiyama, T., Sudo, C., Ogawara, H., Toyoshima, K. and Yamamoto, T. The product of the human c-*erb*B-2 gene: a 185-kilodalton glycoprotein with tyrosine kinase activity. *Science,* **232,** 1644–1646 (1986).
22) Tsuda, H., Hirohashi, S., Shimosato, Y., Hirota, T., Tsugane, S., Watanabe, S., Terada, M. and Yamamoto, H. Correlation between histologic grade of malignancy and copy number of c-erbB-2 gene in breast carcinoma. A retrospective analysis of 176 cases. *Cancer,* **65,** 1794–1800 (1990).
23) Yamazaki, H., Fukui, Y., Ueyama, Y., Tamaoki, N., Kawamoto, T., Taniguchi, S. and Shibuya, M. Amplification of the structurally and functionally altered epidermal growth factor receptor gene (*c-erb*B) in human brain tumors. *Mol. Cell Biol.,* **8,** 1816–1820 (1988).
24) Zabrecky, J. R., Lam, T., McKenzie, S. R. and Carney, W. The extracellular domain of p185/*neu* is released from the surface of human breast carcinoma cells, SK-BR-3. *J. Biol. Chem.,* **266,** 1716–1720 (1991).
25) Ramachandra, S., Machin, L., Ashley, S., Monaghan, P. and Gusterson, B. A. Immunohistochemical distribution of c-erbB-2 in *in situ* breast carcinoma — a detailed morphological analysis. *J. Pathol.,* **161,** 7–14 (1990).
26) Tsutsumi, Y., Naber, S. P., DeLellis, R. A., Wolfe, H. J., Marks, P. J., McKenzie, S. J. and Yin, S. *neu* oncogene protein and epidermal growth factor receptor are independently expressed in benign and malignant breast tissues. *Hum. Pathol.,* **21,** 750–758 (1990).
27) Tsuda, H., Hirohashi, S., Shimosato, Y., Hirota, T., Tsugane, S., Yamamoto, H., Miyajima, N., Toyoshima, K., Yamamoto, T., Yokota, J., Yoshida, T., Sakamoto, H., Terada, M. and Sugimura, T. Correlation between long-term survival in breast cancer patients and amplification of two putative oncogene-coamplification units: *hst*-1/*int*-2 and c-*erb*B-2/*ear*-1. *Cancer Res.,* **49,** 3104–3108 (1989).
28) Mittra, I. and MacRae, K. D. A meta-analysis of reported correlations between prognostic factors in breast cancer: does axillary lymph node metastasis represent biology or chronology? *Eur. J. Cancer,* **27,** 1574–1583 (1991).
29) Adnane, J., Gaudray, P., Simon, M. P., Simony, L. J., Jeanteur, P. and Theillet, C. Proto-oncogene amplification and human breast tumor phenotype. *Oncogene,* **4,** 1389–1395 (1989).
30) Zeillinger, R., Kury, F., Czerwenka, K., Kubista, E., Sliutz, G., Knogler, W., Huber, J., Zielinski, C., Reiner, G., Jakesz, R., Staffen, A., Reiner, A., Wrba, F. and Spona, J. HER-2 amplification, steroid receptors and epidermal growth factor receptor in primary breast cancer. *Oncogene,* **4,** 109–114 (1989).
31) Yamada, Y., Yoshimoto, M., Murayama, Y., Ebuchi, M., Mori, S., Yamamoto, T., Sugano, H. and Toyoshima, K. Association of elevated expression of the c-*erb*B-2 protein with spread of breast cancer. *Jpn. J. Cancer Res.,* **80,** 1192–1198 (1989).
32) Uehara, T., Kaneko, Y., Kanda, N., Yamamoto, T., Higashi, Y., Nomoto, C., Izumo, T., Takayama, S. and Sakurai, M. c-*erb*B-2 and c-*erb*A-1 (*ear*-1) gene amplification and c-*erb*B-2 protein expression in Japanese breast cancers: their relationship to the histology and other disease parameters. *Jpn. J. Cancer Res.,* **81,** 620–624 (1990).
33) Berns, E. M., Klijn, J. G., van Staveren, I. L., Portengen,

H., Noordegraaf, E. and Foekens, J. A. Prevalence of amplification of the oncogenes c-*myc*, *HER2/neu*, and *int*-2 in one thousand human breast tumours: correlation with steroid receptors. *Eur. J. Cancer,* **28,** 697–700 (1992).
34) Read, L. D., Keith, D. J., Slamon, D. J. and Katzenellenbogen, B. S. Hormonal modulation of HER-2/*neu* protooncogene messenger ribonucleic acid and p185 protein expression in human breast cancer cell lines. *Cancer Res.,* **50,** 3947–3951 (1990).
35) Russell, K. C. and Hung, M.-C. Translational repression of the neu protooncogene by estrogen stimulated estrogen receptor. *Cancer Res.,* **52,** 6624–6629 (1992).
36) De Bortoli, M., Dati, C., Antoniotti, S., Maggiora, P. and Sapei, M. L. Hormonal regulation of *c-erb*B-2 oncogene expression in breast cancer cells. *J. Steroid Biochem. Mol. Biol.,* **43,** 21–25 (1992).
37) Hancock, M. C., Langton, B. C., Chan, T., Toy, P., Monahan, J. J., Mischak, R. P. and Shawver, L. K. A monoclonal antibody against the c-*erb*B-2 protein enhances the cytotoxicity of *cis*-diamminedichloroplatinum against human breast and ovarian tumor cell lines. *Cancer Res.,* **51,** 4575–4580 (1991).