Jpn. J. Cancer Res. 80, 1192–1198, December 1989

Association of Elevated Expression of the c-*erbB*-2 Protein with Spread of Breast Cancer

Yukinori Yamada,[1] Masataka Yoshimoto,[2] Yoichi Murayama,[3] Masakazu Ebuchi,[4] Shigeo Mori,[5] Tadashi Yamamoto,[1] Haruo Sugano[6] and Kumao Toyoshima[1,7]

Departments of [1]Oncology and [5]Pathology, Institute of Medical Science, University of Tokyo, 4-6-1, Shirokanedai, Minato-ku, Tokyo 108, [2]Department of Surgery, Cancer Institute Hospital and [6]Cancer Institute, 1-37-1, Kami-Ikebukuro, Toshima-ku, Tokyo 170, [3]Second and [4]First Departments of Surgery, Tokyo Medical and Dental University, 1-5-45, Yushima, Bunkyo-ku, Tokyo 113

We analyzed amplification and expression of the c-*erbB*-2 gene in human breast cancers. Southern blot hybridization analysis demonstrated amplification of the c-*erbB*-2 gene in 10 out of 50 tumor DNAs examined. The degree of amplification was three- to twenty-fold relative to normal placenta. The c-*erbB*-2 protein could be analyzed in 39 tumor tissues of the 50 samples by immuno-blotting, and elevated expression of the c-*erbB*-2 protein was found in 15 cases. On the other hand, expression of the c-*erbB*-2 products was not detected in normal breast tissues either by immuno-blotting or by immuno-histological analysis. These data indicate that transcriptional and/or translational activation of c-*erbB*-2 might occur in some breast cancers in addition to activation by gene amplification. The elevated expression of the c-*erbB*-2 protein was most strongly correlated with lymph-node metastasis ($P<0.001$), suggesting that c-*erbB*-2 expression is involved in promotion of the lymph-node metastasis of human breast cancers. Therefore, immuno-histological diagnosis with anti-c-*erbB*-2 antibody might be useful as an indicator to predict lymph-node involvement in breast cancer.

Key words: c-*erbB*-2 — Gene amplification — Overexpression — Breast cancer — Lymph-node metastasis

The molecular mechanism of the initiation and progression of cancer is still largely unknown. In recent years, however, a major goal of cancer research has been to understand the disease as a disease of the genes and to correlate alterations of various proto-oncogenes and possible tumor suppressor genes with pathological and clinical features of tumors. For example, amplification of N-*myc* was correlated with the stage of neuroblastoma,[1,2] and amplification of the epidermal growth factor (EGF) receptor gene, the c-*erbB* proto-oncogene, was frequently observed in human squamous-cell carcinomas.[3]

The c-*erbB*-2/HER-2 gene encodes a glycoprotein structurally similar to, but distinct from, the EGF receptor,[4,5] and its product is associated with tyrosine kinase activity, and is expected to play roles in growth control of cells just like other growth factor receptors.[6] However, no ligand for the c-*erbB*-2 product has been identified.

In breast cancer, which is one of the main causes of death from cancer among women, a proto-oncogene c-*erbB*-2 has been found to be frequently amplified.[7–15] Two mechanisms are mainly suspected to play a role in oncogenic activation of the c-*erbB*-2 gene. One is the overexpression of its product,[16,17] and the other is a point mutation from Val^{664} to Glu in the transmembrane region,[18] as occurs in the *neu* gene, which is rat counterpart of the c-*erbB*-2 gene. However, the c-*erbB*-2 gene requires two successive mutations in a single codon to acquire transforming capacity by the same amino acid substitution and such successive mutations would be extremely rare. On the other hand, amplification of the c-*erbB*-2 gene has been observed in 10 to 30% of the DNA samples prepared from breast tumors.[8–15] In addition, amplification of c-*erbB*-2 was reportedly linked to clinical features such as poor prognosis of the patients, and lymph-node metastasis of the tumor. However, conflicting data as to the correlations between gene amplification and poor prognosis were also reported.[14] Concerning breast cancer in Japanese patients, c-*erbB*-2 does seem to be involved in the malignancy of the breast cancers, as shown in a recent report of a retrospective study using 176 patients with breast cancer.[15]

To examine the possibility that elevated expression of the c-*erbB*-2 product is associated with the breast cancer progression, we analyzed the level of c-*erbB*-2 expression in tumors by immuno-blotting and immuno-histological study in addition to Southern hybridization.

We show here that elevated expression of the c-*erbB*-2 protein occurs more frequently than gene amplification in breast cancers. In addition, the degree of lymph-node metastasis was significantly correlated with c-*erbB*-2 gene amplification as well as elevated expression of the gene product. The clinical stage was also correlated with the c-*erbB*-2 expression. These results are consistent with the

[7] To whom correspondence should be addressed.

hypothesis that the c-*erbB*-2 protein expression is one of the dominant parameters influencing the progression of breast cancer.

MATERIALS AND METHODS

Surgical specimens Tumors were taken from patients with breast cancer undergoing surgical operations between 1985 and 1988. The primary tumors were analyzed in 50 cases and among these cases, metastatic lymph nodes were available for analysis in 5 cases. All tissues were stored at −70°C before use.

Southern hybridization High-molecular-weight DNA was extracted from tumor tissues and then digested with a restriction endonuclease, *Eco*RI, *Hin*dIII or *Bam*HI. The digests (10 μg) were subjected to electrophoresis on 1.0% agarose gel. The fractionated DNAs were denatured and transferred to nitrocellulose filters. The 4.4 kb *Dra*I-*Dra*I fragment of human c-*erbB*-2 complementary DNA and the 2.4 kb *Cla*I-*Cla*I fragment of EGF receptor complementary DNA were labeled with ^{32}P by using a multi-primed labeling system (Amersham). The filters were hybridized with the ^{32}P-labeled c-*erbB*-2 or EGF receptor DNA (specific activity 2×10^8 cpm/μg) under stringent conditions, washed, dried and exposed to Kodak XAR-5 films.

Immuno-blotting Frozen tissues were minced and homogenized in RIPA buffer (50 m*M* Tris-HCl (pH 7.4), 150 m*M* NaCl, 1% NP-40, 0.1% sodium deoxycholate, 1 m*M* phenylmethylsulfonyl fluoride, 200 U/ml aprotinin). These lysates were measured by Bradford assay[19] and 100 μg of total protein lysate was subjected to electrophoresis on 7.5% sodium dodecyl sulfate polyacrylamide gel. The fractionated proteins were electro-transferred to nitrocellulose filters, and the filters were blocked in 2% skimmed milk/Tris-buffered saline, and reacted with the polyclonal anti c-*erbB*-2 rabbit antibody against the C-terminal 14 peptides.[6] After the proteins on the filters were bound to the first antibody, the filters were reacted with the second antibody of the Proto-Blot Immunoblotting System (Promega Biotec), washed, and processed for the color reaction according to the procedure recommended by the supplier.

Immuno-staining Frozen sections were cut with a cryostat and fixed on slides with 4% paraformaldehyde in 0.1 *M* phosphate buffer at pH 7.4 for 30 min. They were then washed with phosphate-buffered saline (PBS) for 10 min. For blocking of endogenous avidin-binding activity (EABA), an EABA-blocking kit (Vector Laboratories) was used. The slides were incubated with appropriately diluted avidin solution for 5 min, washed, and then incubated with biotin solution for 5 min according to the supplier's instructions. The slides were then subjected to immuno-staining following the method of Hsu *et al.*[20] with slight modifications as below. A Vectastain ABC kit (Vector Laboratories) was used as the second and third-phase reagents for immuno-staining. The slides were incubated with appropriately diluted first antibody (1:50 for anti c-*erbB*-2) for 30 min. They were then washed with phosphate-buffered saline, incubated with the second and third reagents for 30 min, respectively, and washed. Finally, they were stained with 0.6 mg/ml 3,3′-diaminobenzidine tetrahydrochloride in PBS containing 0.01% H_2O_2 for 5 min and mounted.

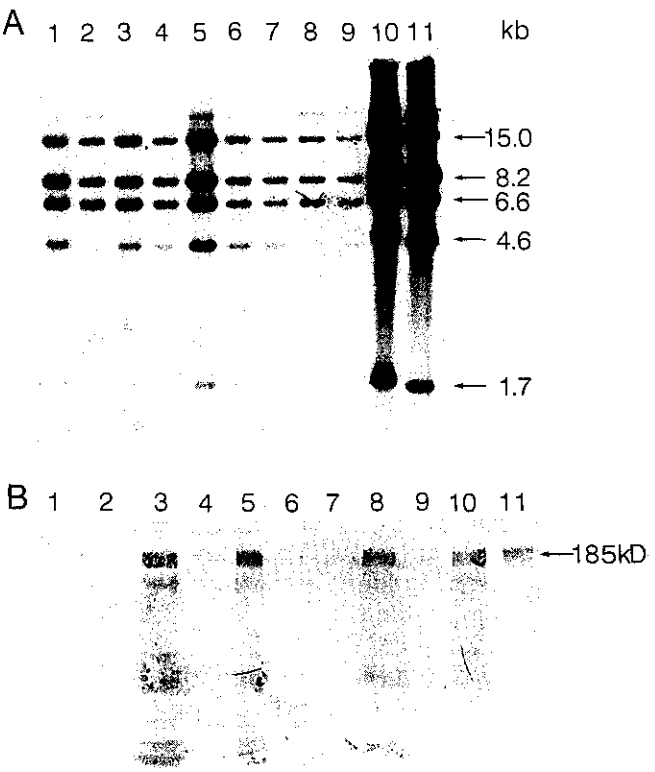

Fig. 1. A. Southern hybridization analysis. High-molecular-weight DNAs were extracted from breast cancers, digested with *Eco*RI and hybridized with c-*erbB*-2 cDNA probe. Lane 1, DNA from normal human placenta; lanes 2–10 DNAs isolated from human breast carcinomas; lane 11, DNA from human adenocarcinoma cell line, MKN7, which shows approximately 30-fold amplification of c-*erbB*-2 gene relative to placenta. The sizes of the fragment are shown in kb on the right. B. Immuno-blotting analysis of the lysates derived from breast cancers. A sample of 50 μg of protein was electrophoresed in a 7.5% SDS-polyacrylamide gel. Fractionated proteins were transferred onto nitrocellulose filters, which were then reacted with anti c-*erbB*-2 polyclonal antibodies. Lane 1, the lysates from normal mammary glands; lanes 2–11 correspond to lanes 2–11 in Fig. 1A. The sizes of c-*erbB*-2 proteins are indicated on the right.

RESULTS

Fifty primary breast tumors and 4 metastatic lymph nodes were examined for alterations of the c-*erbB*-2 gene. By Southern blot hybridization with the c-*erbB*-2 probe, 5 *Eco*RI fragments of 15.0, 8.2, 6.6, 4.6, and 1.7 kilobase pairs (kbp), but no additional band, were detected in all the samples and in a control placenta DNA, showing that the c-*erbB*-2 gene was not grossly rearranged in these tumors (Fig. 1A). However, the signals corresponding to the c-*erbB*-2 specific fragments in some of the tumors were more intense than in normal placenta, indicating the presence of higher copy numbers of the c-*erbB*-2 gene in these tumors (Fig. 1A lanes 5 and 10). Densitometric assay indicated that the degree of amplification was three- to twenty-fold in these tumors compared with the control sample. Six out of ten samples, which showed amplification of the c-*erbB*-2 gene, were scirrhous-type carcinomas (Table I). After removal of the first probe, the same filters were hybridized with the EGF receptor cDNA probe; however, this probe detected neither rearrangement not amplification of the EGF receptor gene in any of the tissues (data not shown).

We then analyzed expression of the c-*erbB*-2 gene product by immuno-blot analysis with anti-c-*erbB*-2 polyclonal antibody. The specificity of this antibody in immunoblotting was demonstrated previously by competition experiments with synthetic peptides.[6] Cell lysates were prepared from 39 tissues out of the 50 samples examined above and 15 showed high expression of the c-*erbB*-2 protein, $gp185^{erbB2}$. No expression of $gp185^{erbB2}$ was observed in apparently normal mammary tissues from regions adjecent to the breast cancer (Fig. 1B lane 1). The samples with c-*erbB*-2 gene amplification were always found to express $gp185^{erbB2}$ (Fig. 1 lanes 5 and 10). Furthermore, 6 tumors without amplification of the c-*erbB*-2 gene were proved to show high expression of $gp185^{erbB2}$ (Fig. 1 lanes 3 and 8). These data suggest that the c-*erbB*-2 expression in some breast cancer tissues is upregulated at the level of transcription and/or translation. There was no evidence that aberrantly sized c-*erbB*-2 products were expressed in any of these samples.

The immuno-histochemical study showed that the anti-c-*erbB*-2 polyclonal antibody reacted with cancer cells, especially in the apical site when expression was high, but not with the normal cells in the same specimen (Fig. 2B, C). In addition, none of the cells in normal mammary glands was stained with anti-c-*erbB*-2 antibody (Fig. 2A). The results from the immuno-staining are consistent with the immuno-blot analysis.

Histological types of breast cancers that showed amplification and overexpression of the c-*erbB*-2 gene are summarized in Table I. The frequencies of both amplification and overexpression of the c-*erbB*-2 were high in scirrhous-type carcinomas compared with the other types. Then, we investigated the correlation between c-*erbB*-2 and clinical parameters of the breast cancers, such as the degree of lymph-node metastasis, the presence of receptors for estrogen and progesterone, clinical TNM stage,[21] tumor size, age and status of menopause. Among these factors, the degree of lymph-node metastasis was correlated with c-*erbB*-2 gene amplification ($P<0.01$). Although Pearson's χ^2 statistics (P values) were not significant, tumor tissues that are positive for estrogen receptor at stages III and IV or larger than 5 cm in diameter tend to carry amplified c-*erbB*-2 gene (Table II). There was no correlation between c-*erbB*-2 amplification and the other parameters (data not shown). When the correlation between the clinical parameters and the protein expression was analyzed (Table III), similar results were obtained. The degree of lymph-node metastasis was more strongly linked to the expression of the c-*erbB*-2 gene product ($P<0.001$) than to the c-*erbB*-2 gene amplification. Furthermore, correlation between the clinical TNM stage and expression of the c-*erbB*-2 gene product was significant ($P<0.01$). The relationship between the clinical parameters and amplification/expression is summarized in Table IV.

Table I. Frequency of c-*erbB*-2 Amplification/Overexpression in Breast Cancers

Tumors	No. of amplification/ No. of total cases (%)	No. of overexpression/ No. of total cases (%)
Primary tumor	10/50 (20)	15/39 (38)
Papillary tubular ca.	1/9 (11)	1/7 (14)
Solid tubular ca.	3/17 (18)	5/14 (36)
Scirrhous ca.	6/20 (30)	9/16 (56)
Other type	0/4 (0)	0/2 (0)
Metastatic lymph node	2/4 (50)	1/2 (50)

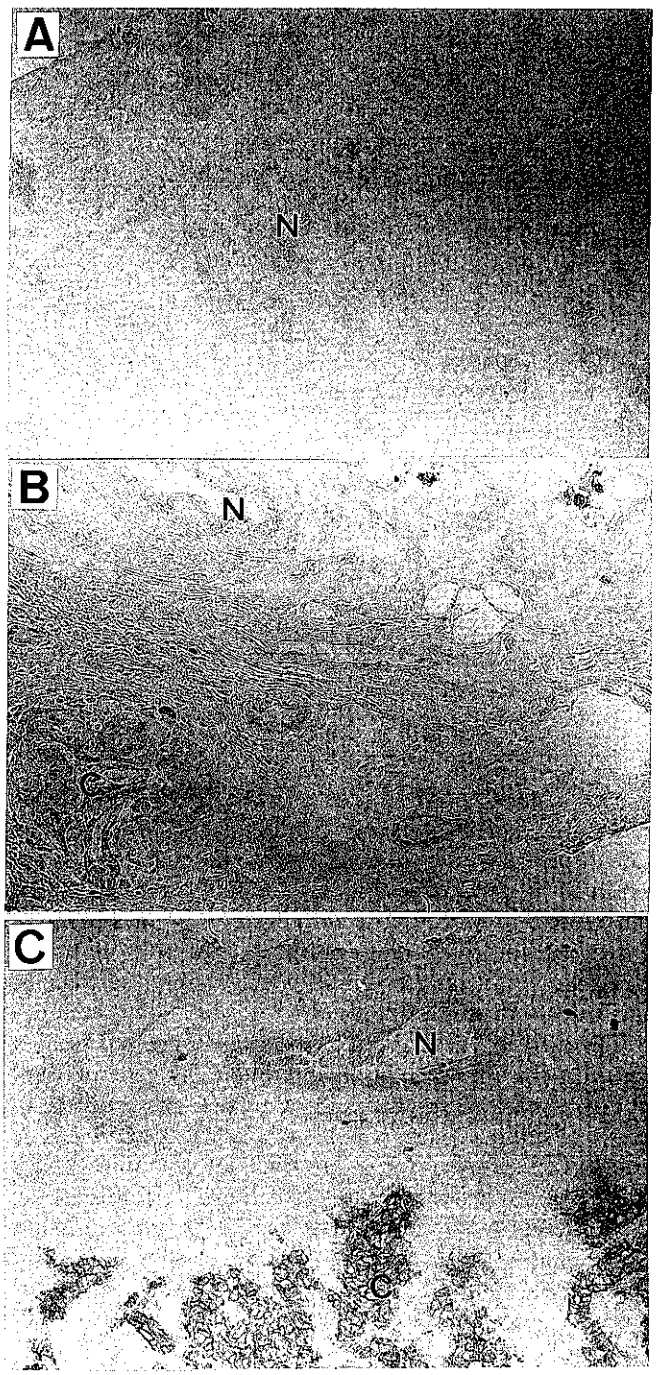

Fig. 2. Immuno-staining analysis of human breast carcinomas and normal mammary gland tissues with anti c-*erbB*-2 polyclonal antibodies. A: normal mammary glands (non-stained). B: scirrhous type mammary carcinomas (weakly stained). C: solid tubular type mammary carcinomas (strongly stained). N indicates a normal mammary gland and C represents carcinoma cells.

DISCUSSION

Amplification of the c-*erbB*-2 gene is thought to be correlated with poor prognosis and spread of breast cancer, suggesting that elevated expression of the c-*erbB*-2 gene is involved in these clinical behaviors.[8–13, 15] It was also reported that elevated expression of the c-*erbB*-2 protein resulted in malignant transformation of phenotypically normal cells *in vitro*.[16, 17] These data together suggest that elevated expression of the c-*erbB*-2 gene is involved in development and/or progression of the tumor. However, there are some conflicting reports that the c-*erbB*-2 expression showed no apparent correlation with lymph-node involvement or with relapse-free survival of breast cancer.[14] To date, no reasonable explanation for this discrepancy has been available. Most of the previous studies dealt with amplification of the gene in the breast cancer and relatively few studied expression of the c-*erbB*-2 protein immuno-histochemically. Therefore, in order to establish the correlation between clinical parameters and c-*erbB*-2 expression, we analyzed 50 breast carcinoma tissues by Western and Southern blottings. As previously reported, Southern blot analysis showed amplification of the c-*erbB*-2 gene in 20% of the cases. In addition, c-*erbB*-2 overexpression, analyzed by immuno-blots using anti-c-*erbB*-2 antibody, was seen in as many as 38% of the cases. In this analysis, the samples with amplified c-*erbB*-2 gene invariably showed elevated expression of the gene product. Consistent with our observation, the c-*erbB*-2 mRNA was reported to be overexpressed in 36% of the breast carcinomas.[13] These data suggest that overexpression of the c-*erbB*-2 gene in breast cancer is partly regulated by a transcriptional mechanism other than by gene amplification, as pointed out by Guerin *et al.*

Our data also showed that both tumor stage and lymph-node metastasis were correlated with amplification of the gene as well as expression of the gene product. A closer correlation was noted between lymph-node metastasis and c-*erbB*-2 protein expression. Expression of the c-*erbB*-2 protein was specifically detected in tumor cells, but only a little expression of the c-*erbB*-2 protein was seen in normal tissue of the breast (Fig. 2). Among 15 cases of c-*erbB*-2 overexpression, all but one case were proved to be associated with apparent lymph-node metastasis. Thus, we assume that elevated expression of the c-*erbB*-2 protein may be useful as a diagnostic marker for invasion of breast cancer. These results indicate that rapid diagnosis by immuno-staining would be useful for the correct indication of extended surgical operation or post-operative adjuvant therapy.

We have previously reported that the c-*erbB*-2 gene is amplified in 14% of stomach cancers.[22] We further

Table II. Association between c-*erbB*-2 Amplification and Disease Parameters in Breast Cancers

Factor		Not amplified	Amplified	Total	*P*
Estrogen	+	23	6	29	NS
receptor	−	13	1	14	
TNM stage	I and II	31	4	35	NS
	III and IV	9	6	15	
Tumor size	≦5 cm	35	6	41	NS
	>5 cm	4	4	8	
Lymph-node	n_0n_1	35	4	39	<0.01
metastasis	n_2n_3	5	6	11	

Table III. Association between c-*erbB*-2 Expression and Disease Parameters in Breast Cancers

Factor		Not expressed	Expressed	Total	*P*
Estrogen	+	11	10	21	NS
receptor	−	11	3	14	
TNM stage	I and II	20	5	25	<0.01
	III and IV	4	10	14	
Tumor size	≦5 cm	20	11	31	NS
	>5 cm	3	4	7	
Lymph-node	n_0n_1	22	6	28	<0.001
metastasis	n_2n_3	2	9	11	

Table IV. Distribution of c-*erbB*-2 Amplification and Expression in Disease Parameters

	Stage			
	I	II	III	IV
Amplification	0/6	4/29	3/12	3/3
Expression	0/4	5/21	7/11	3/3

	Tumor size		
	<2 cm	2–5 cm	>5 cm
Amplification	0/4	6/37	4/8
Expression	1/2	10/29	4/7

	Lymph-node metastasis			
	n_0	n_1	n_2	n_3
Amplification	0/19	4/20	2/6	4/5
Expression	1/10	5/18	4/6	5/5

showed that amplification of the c-*erbB*-2 gene is confined to adenocarcinomas, especially tubular adenocarcinomas of the stomach. We also noted that amplification of the EGF receptor gene occurred more frequently in well differentiated type of squamous carcinomas than in poorly differentiated type of squamous carcinomas.[3] In breast cancer, amplification of the c-*erbB*-2 gene was more frequent in scirrhous carcinomas than in tubular adenocarcinomas. Scirrhous carcinoma cells have properties of poorly differentiated glandular epithelial cells. Therefore, it seemed that amplification of these growth factor receptor-encoding genes plays a role in tumor development or progression of poorly differentiated cells, in contrast to stomach cancer.

The c-*erbB*-2 gene product has been suggested to be a receptor for an unknown ligand. Since the gene product is expressed in epithelial cells of fetal tissues but not in those of adult tissues, it is likely that the c-*erbB*-2 gene encodes a receptor for a growth factor that regulates proliferation of embryonal epithelium.[23] If this is the case, the growth factor may not be present in adult tissues and c-*erbB*-2 gene product expressed in mammary tumors would not be receiving signals unless the tumor cells produce the growth factor. In addition, previous transfection assay of DNAs from 8 patients with breast cancer did not reveal any transforming ability of c-*erbB*-2 (Cline and Yamamoto, unpublished data). Thus, we assume that elevated tyrosine kinase activity in tumor cells expressing normal c-*erbB*-2 at the elevated level may contribute to tumor formation and/or progression.

Recently, during the preparation of this paper, papers which support our results were reported Lacroix *et al.*[24] and Slamon *et al.*[25] Both reports were consistent with

our data in that a discrepancy was observed between c-*erbB*-2 gene amplification and protein expression. Also, they proposed a relationship between the protein expression and poor prognosis of the patients. In the former report, c-*erbB*-2 protein expression was observed to be more strongly correlated with the inflammatory carcinomas than with the lymph-node status. However, inflammatory carcinomas with c-*erbB*-2 overexpression may act as aggressively growing tumor cells, which should promote the spread of cancer cells locally or at a distance, as discussed by Lacroix *et al.*[24]

ACKNOWLEDGMENTS

This research was supported in part by a Grant-in-Aid for Special Project Research from the Ministry of Education, Science and Culture of Japan.

(Received July 22, 1989/Accepted October 30, 1989)

REFERENCES

1) Schwab, M. Amplification of N-*myc* in human neuroblastomas. *Trend Genet.*, **1**, 271–275 (1985).
2) Seeger, R. C., Brodeur, G. M., Sather, H., Dalton, A., Siegel, S. E., Wong, K. Y. and Hammond, D. Association of multiple copies of the N-*myc* oncogene with rapid progression of neuroblastomas. *N. Eng. J. Med.*, **313**, 1111–1116 (1985).
3) Cowley, G., Smith, J. A., Gusterson, B., Hendler, F. and Ozanne, B. The amount of EGF receptor is elevated on squamous cell carcinomas. *Cancer Cells*, **1**, 5–10 (1984).
4) Semba, K., Kamata, N., Toyoshima, K. and Yamamoto, T. A v-*erbB*-related protooncogene, c-*erbB*-2, is distinct from the c-*erbB*-1/epidermal growth factor receptor gene and is amplified in a human salivary gland adenocarcinoma. *Proc. Natl. Acad. Sci. USA*, **82**, 6497–6501 (1985).
5) Yamamoto, T., Ikawa, S., Akiyama, T., Semba, K., Nomura, N., Miyajima, N., Saito, T. and Toyoshima, K. Similarity of protein encoded by the human c-*erbB*-2 gene to epidermal growth factor receptor. *Nature*, **319**, 230–234 (1986).
6) Akiyama, T., Sudo, C., Ogawara, H., Toyoshima, K. and Yamamoto, T. The product of human c-*erbB*-2 gene: a 185-kilodalton glycoprotein with tyrosine kinase activity. *Science*, **232**, 1644–1646 (1986).
7) King, C. R., Kraus, M. H. and Aaronson, S. A. Amplification of a novel v-*erbB*-related gene in a human mammary carcinoma. *Science*, **229**, 974–976 (1985).
8) Slamon, D. J., Clark, G. M., Wong, S. G., Levine, 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).
9) Kraus, M. H., Popscu, N. C., Amsbaugh, S. C. and King, C. R. Overexpression of the EGF receptor-related protooncogene *erbB*-2 in human mammary tumor cell lines by different molecular mechanisms. *EMBO J.*, **6**, 605–610 (1987).
10) Zhou, D., Battifora, H., Yokota, J., Yamamoto, T. and Cline, M. J. Association of multiple copies of the c-*erbB*-2 oncogene with spread of breast cancer. *Cancer Res.*, **47**, 6123–6125 (1987).
11) Tal, M., Wetzler, M., Josefberg, Z., Deutch, A., Gutman, M., Assaf, D., Kris, R., Shiloh, Y., Givol, D. and Schlessinger, J. Sporadic amplification of the *HER2/neu* protooncogene in adenocarcinomas of various tissues. *Cancer Res.*, **48**, 1517–1520 (1988).
12) Vijver, M., Bersselaar, R., Devilee, P., Cornelisse, C., Peterse, J. and Nusse, R. Amplification of the *neu*(c-*erbB*-2) oncogene in human mammary tumors is relatively frequent and is often accompanied by amplification of the linked c-*erbA* oncogene. *Mol. Cell. Biol.*, **7**, 2019–2023 (1987).
13) Guerin, M., Barrois, M., Terrier, M. J., Spielmann, M. and Riou, G. Overexpression of either c-*myc* or c-*erbB*-2/*neu* proto-oncogenes in human breast carcinomas: correlation with poor prognosis. *Oncogene Res.*, **3**, 21–31 (1988).
14) Ali, I. U., Cambell, G., Lidereau, R. and Callahan, R. Lack of evidence for the prognostic significance of c-*erbB*-2 amplification in human breast carcinoma. *Oncogene Res.*, **3**, 139–146 (1988)
15) 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-*erbB*-2/*ear*-1. *Cancer Res.*, **49**, 3104–3108 (1989).
16) Hudziak, R. M., Schlessinger, J. and Ullrich, A. Increased expression of the putative growth factor receptor $p185^{HER2}$ causes transformation and tumorigenesis of NIH3T3 cells. *Proc. Natl. Acad. Sci. USA*, **84**, 7159–7163 (1987).
17) DiFiore, P. P., Pierce, J. H., Kraus, M. H., Seggato, O., King, C. R. and Aaronson, S. A. *ErbB*-2 is a potent oncogene when overexpressed in NIH3T3 cells. *Science*, **237**, 178–182 (1987).
18) Bargmann, C. I., Hung, M. C. and Weinberg, R. A. Multiple independent activations of the *neu* oncogene by a point mutation altering the transmembrane domain of p185. *Cell*, **45**, 649–657 (1986).
19) Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72**, 248–254 (1976).
20) Hsu, S. M., Rayhe, L. and Fanger, H. Use of avidin-biotin-peroxidase complex (ABC) in immuno-peroxidase

techniques. *J. Histochem. Cytochem.*, **29**, 577–580 (1981).
21) TNM General Rules 2nd Ed. (1974). VICE, Geneva.
22) Yokota, J., Yamamoto, T., Miyajima, N., Toyoshima, K., Nomura, N., Sakamoto, H., Yoshida, T., Terada, M. and Sugimura, T. Genetic alterations of the c-*erbB*-2 oncogene occur frequently in tubular adenocarcinoma of the stomach and are often accompanied by amplification of the v-*erbA* homologue. *Oncogene*, **2**, 283–287 (1988).
23) Mori, S., Akiyama, T., Yamada, Y., Morishita, Y., Sugawara, I., Toyoshima, K. and Yamamoto, T. C-erbB-2 gene product, a membrane protein commonly expressed on human fetal epithelial cells. *Lab. Invest.*, **61**, 93–97 (1989).
24) Lacroix, H., Iglehart, J. D., Skinner, M. A. and Kraus, M. H. Overexpression of *erbB*-2 or EGF receptor proteins present in early stage mammary carcinoma is detected simulataneously in matched primary tumors and regional metastasis. *Oncogene*, **4**, 145–151 (1989).
25) Slamon, D. J., Godolphin, W., Jones, L. A., Holt, J. A., Wong, S. G., Keith, D. E., Levine, W. J., Stuart, S. G., Udove, J., Ullrich, A. and Press, M. F. Studies of the HER-2/*neu* proto-oncogene in human breast and ovarian cancer. *Science*, **244**, 707–711 (1989).