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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

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Using a polyclonal antibody monospecific to the c-*erb*B-2 oncogene product, an immunohistochemical study on the expression of c-*erb*B-2 protein was performed in formalin-fixed, paraffin-embedded tissue sections from 176 primary breast carcinomas in which amplification of the c-*erb*B-2 gene had been detected in 28 cases. Expression of the c-*erb*B-2 protein was detected in 44 cases (25%), being strongly positive in 27 (15%) and weakly positive in 17 (10%). All cases with amplification of c-*erb*B-2 showed positive staining of its protein. There were only four cases in which c-*erb*B-2 was strongly expressed without amplification of the gene. In the group showing strongly positive staining, both overall and disease-free survival were significantly poorer than in the remainder of the cases. Using Cox's regression model analysis, overexpression of c-*erb*B-2 protein was demonstrated to be an effective prognostic factor independent of nodal status or tumor size.

Key words: Human breast cancer — c-*erb*B-2 protein — Amplification of c-*erb*B-2 gene — Immunohistochemistry — Prognostic factor

The human c-*erb*B-2 gene, which is identical to the *neu*, *HER*-2 and *NGL* genes, has been isolated as a new oncogene homologous with but distinct from the c-*erb*B or EGFR (epidermal growth factor receptor) gene.[1-4] The product of the c-*erb*B-2 gene is a 185-kilodalton glycoprotein composed of cytoplasmic, transmembrane and extracellular domains, and its amino acid sequence homology with EGFR indicates that the gene also encodes a growth factor receptor with tyrosine-kinase activity.[5] However, the ligand for this receptor protein is still unknown. The c-*erb*B-2 gene is often amplified in adenocarcinomas of the breast,[6,7] stomach,[8] and ovary.[7] In studies of breast cancer, amplification of the c-*erb*B-2 gene has been shown to be associated with poorer prognosis of the patients.[6,7,9] There are also reports showing that overexpression of c-*erb*B-2 mRNA or c-*erb*B-2 protein is a prognostic indicator in cases of human breast cancer.[10,11]

We showed previously that breast carcinoma patients with amplification of the c-*erb*B-2 gene in the primary tumor had a poorer prognosis.[9] In the present study, we examined the correlation between expression of c-*erb*B-2 protein and amplification of the c-*erb*B-2 gene itself, or the long-term prognosis of patients by immunohistochemical study of identical tissue blocks of breast carcinoma from which DNA had been isolated in the previous study.[9] The possible use of immunohistochemistry for routine examination of c-*erb*B-2 expression was also discussed.

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MATERIALS AND METHODS

Patients The 176 patients analyzed in this study were the same as those described in the previous paper.[9] Data on age, menstrual status, tumor size, lymph nodal status of metastasis and 10-year prognosis after initial surgical treatment were available. Formalin-fixed, paraffin-embedded tissue blocks of the lesions of primary breast carcinoma which were identical to those used in the previous study on amplification of the c-*erb*B-2 gene were sliced into sections 3 μm thick.

Immunohistochemistry The polyclonal antibody used had been raised in rabbits against a synthetic peptide corresponding to amino acid residues 1,242 to 1,255 (Thr-Ala-Glu-Asn-Pro-Glu-Tyr-Leu-Gly-Leu-Asp-Val-Pro-Val) of the cytoplasmic domain of the human c-*erb*B-2 protein.[5] The polyclonal antibody had already been shown to detect specifically the 185-kilodalton c-*erb*B-2 protein in lysates of breast cancer tissue by immunoblot analysis.[12] Immunohistochemical staining was performed according to the method of Hsu *et al.*[13] Sections were deparaffinized in xylene, and rehydrated in

a descending ethanol series, ending in water. They were then incubated for 30 min in 0.3% hydrogen peroxide in methanol, and preincubated in 2% normal swine serum in phosphate-buffered saline in order to abolish endogenous peroxidase activity and to diminish any non-specific antibody binding. The sections were incubated at 4°C onvernight with the polyclonal antibody against c-*erb*B-2 peptide at a dilution of 1:200, and then incubated for 30 min with biotinylated goat anti-rabbit immunoglobulin as a secondary antibody (Vector Laboratories Inc., Burlingame, CA) diluted 1:200. Subsequently, they were incubated for 30 min with avidin-biotinyl peroxidase complex using a Vectastain ABC Kit (Vector) diluted 1:100 in phosphate-buffered saline. The peroxidase reaction was performed using 3,3′-diaminobenzidine tetrahydrochloride-hydrogen peroxide as a chromogen in Tris buffer (pH 7.6) for 5–10 min. The sections were counterstained with hematoxylin, dehydrated and mounted. Between each step, the slides were washed three times (5 min each) with phosphate-buffered saline. The specificity of the reaction was confirmed by abolition of staining following preincubation of the antibody with the immunizing peptide (0.2 mg/ml).

Statistical analysis Association between the degree of immunostaining of the c-*erb*B-2 protein and the copy number of the c-*erb*B-2 gene, lymph node status or tumor size was evaluated by chi-squared test. Curves for overall and disease-free survival were drawn according to the Kaplan-Meier method[14] and differences among the curves were calculated by log-rank test.[15] Patients who had undergone palliative surgery were excluded from the disease-free survival curves. The independent prognostic effect of each parameter was computed according to Cox's proportional hazards general linear model using as SAS system package.[16, 17]

RESULTS

Association of overexpression of c-*erb*B-2 protein with clinical and histological parameters The intensity of staining was categorized as follows: −, negative; +, weakly positive; or ++, strongly positive (Fig. 1A–1C). In the strongly positive (++) cases, the surface of the cell membrane was distinctly stained. In the weakly positive group, the staining was faint, and the difference of staining between membrane and cytoplasm was often unclear. Among the total cases, 132 showed negative staining, 17 (10%) showed weakly positive staining and the other 27 (15%) showed strongly positive staining (Table I).

The cases showing strongly positive staining were more frequent among postmenopausal patients than among pre- or perimenopausal patients ($P<0.025$). The mean age of patients tended to be higher in the group

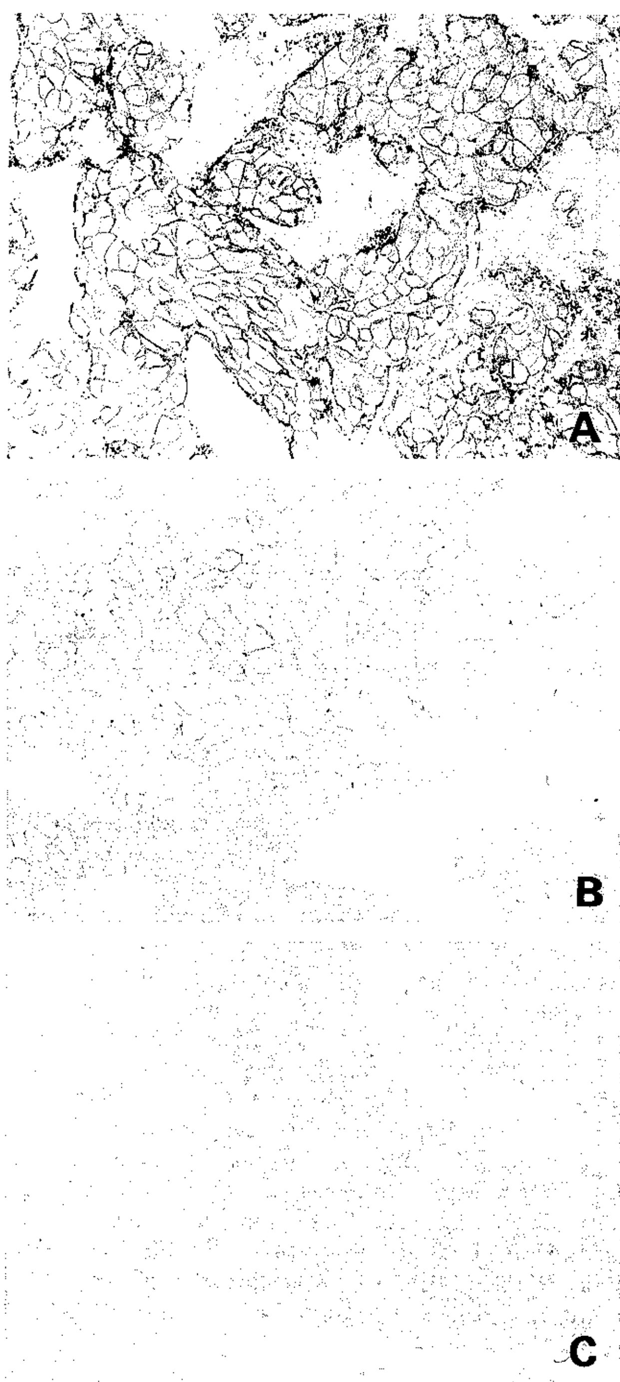

Fig. 1. Photomicrograph of immunostaining of c-*erb*B-2 protein in human breast carcinoma. A. Strongly positive (++) case. Cell surface membrane of all tumor cells is strongly stained. B. Weakly positive (+) case. Cell membrane surface is weakly and only partially stained. C. Negative (−) case. ×200, Immunoperoxidase staining.

Table I. Association of Immunohistochemical Staining of c-*erb*B-2 Protein with Copy Number of c-*erb*B-2 Gene and with Clinical Parameters in Primary Breast Carcinoma

	Number of cases (%)				*P*
	Staining of c-*erb*B-2 protein			Total	
	−	+	++		
A. Copy number of c-*erb*B-2 gene					
<3	132 (89)	12 (8)	4 (3)	148	
3–4	0 (0)	5 (33)	10 (67)	15	<0.001
≥5	0 (0)	0 (0)	13 (100)	13	
B. Menstrual status					
Pre-/perimenopausal	85 (81)	10 (9)	11 (9)	106	<0.025
Postmenopausal	46 (67)	6 (9)	16 (24)	68	
Unknown	1	1	0	2	
C. Number of metastatic lymph nodes					
0	55 (76)	9 (13)	8 (11)	72	
1–3	42 (78)	5 (9)	7 (13)	54	NS
≥4	35 (70)	3 (6)	12 (24)	50	
D. Tumor size on palpation (cm)					
≤2.0	52 (70)	9 (13)	13 (18)	74	
2.1–5.0	61 (80)	8 (11)	7 (9)	76	NS
≥5.1	19 (73)	0 (0)	7 (27)	26	
E. Presence of invasive growth					
Intraductal carcinoma	10 (77)	2 (15)	1 (8)	13	NS
Invasive carcinoma	122 (75)	15 (9)	26 (16)	163	
Total	132 (75)	17 (10)	27 (15)	176	

NS, not significant.

showing strongly positive staining (50.2±10.5 years) than in the group with no or only weak staining (46.1±4.5 years). There was no significant association between intensity of staining and clinical stage, tumor size, lymph node status, or invasive tumor growth (Table I).

Association between overexpression of c-*erb*B-2 protein and amplification of c-*erb*B-2 gene in breast carcinoma The intensity of staining was strongly associated with the copy number of the c-*erb*B-2 gene ($P<0.001$, Table I). All 28 cases of breast carcinoma with amplification of c-*erb*B-2 showed expression of the c-*erb*B-2 protein immunohistochemically, the intensity of staining being strongly positive in 23 cases and weakly positive in 5. There were 4 cases showing strong positivity without amplification of the c-*erb*B-2 gene including one case of intraductal carcinoma.

Correlation between overexpression of c-*erb*B-2 protein and long-term prognosis of breast carcinoma patients There was no difference between the overall survival curves for the group with negative staining of the c-*erb*B-2 protein (132 cases) and the group with weakly positive staining of the protein (17 cases). However, the curve for the group with strong staining of the c-*erb*B-2 protein (27 cases) was significantly different from those of the other two groups ($P<0.001$, Fig. 2, upper). The 10-year survival rate after surgery was 71% in the negative group, 65% in the weakly positive group, and 39% in the strongly positive group. The same tendency was disclosed among disease-free survival curves for the three groups ($P<0.01$, Fig. 2, lower).

Variables listed in Table II were included in the proportional hazards general linear model analysis. Staining of c-*erb*B-2 protein was shown to be an effective prognostic indicator independent of tumor size or lymph node status (Table III).

DISCUSSION

Many immunohistochemical studies have examined the importance of c-*erb*B-2 protein expression as a prognostic factor.[7, 18–22] Some reports have stated that overexpression of the c-*erb*B-2 protein is an important prognostic factor in breast cancer,[7, 10] while others have stated that there is no significant association between overexpression of the gene product and the prognosis of patients.[18–20, 22] We suggest that such a discrepancy

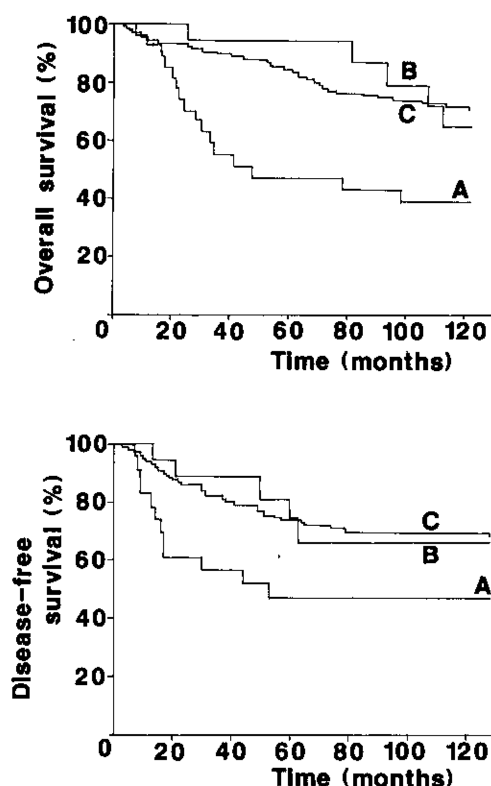

Fig. 2. Survival curves of breast carcinoma patients. Overall survival curves (upper) and disease-free survival curves (lower) for A; the group with strong staining (++) of c-*erb*B-2 protein, B; the group with weak staining (+) of c-*erb*B-2 protein, C; the group with no staining (−) of c-*erb*B-2 protein.

occurred because those reports which denied the association did not take into account the difference between nodal metastasis-negative and -positive patients: we previously found that amplification of c-*erb*B-2 was associated with poor prognosis only in patients with nodal metastasis.[9] Slamon *et al.* examined only node-positive patients and clarified the association between c-*erb*B-2 amplification and expression of its protein with poorer patient prognosis.[6,7] Wright *et al.* also showed a significant difference between the survival curves for node-positive groups with and without positive c-*erb*B-2 immunostaining.[10]

On the other hand, Ali *et al.*[22] and Gusterson *et al.*[19] reported no association of c-*erb*B-2 amplification or overexpression with poor prognosis of breast cancer patients. In those studies,[19,22] however, no analysis of patient lymph nodal status was done. Barnes *et al.*[20] reported that 9% (17/195) of breast cancer cases were strongly positive for c-*erb*B-2 immunostaining and that it had no significant association with poor prognosis. However,

Table II. Variables Included in Proportional Hazards General Linear Model Analysis with Their Respective Scores

Variable	Scores	
Menstrual status	Pre-/perimenopausal	0
	Postmenopausal	1
Tumor size	≤2.0 cm	1
	2.1–5.0 cm	2
	≥5.1 cm	3
Number of metastatic axillary nodes	0	0
	1–3	1
	≥4	2
	Metastasis beyond axillary nodes	3
Staining of c-*erb*B-2 protein	Negative (−)	0
	Weakly positive (+)	1
	Strongly positive (++)	2
Surgical therapy	Curative surgery	0
	Palliative surgery	1
Adjuvant chemotherapy	Not done	0
	Done	1

Table III. Proportional Hazards General Linear Model Comparing Overall Survival with Prognostic Factors in Breast Carcinoma Patients

Factors	Beta	Standard error		*P* value
Number of metastatic lymph nodes	0.856	0.203	17.8	<0.0001
c-*erb*B-2 protein	0.381	0.167	5.2	0.02
Tumor size	0.433	0.247	3.1	0.04
Surgical therapy	0.817	0.514	2.5	0.11
Menstrual status	−0.405	0.320	1.6	0.21
Adjuvant chemotherapy	−0.006	0.438	0.0	0.99

among the 17 positive cases, 12 were free of lymph nodal metastasis.[20] Van de Vijver *et al.*[18] examined immunohistochemically the incidence of c-*erb*B-2 overexpression in 189 breast cancer patients at Stage II. Overexpression was detected in 27 cases (14%), and was not associated with tumor recurrence. However, among the 27 positive cases at Stage II, 10 were free of nodal metastasis.[18] These cases also included 5 tumors with a good prognosis, i.e., 3 ductal carcinomas *in situ* and 2 invasive lobular carcinomas, although their lymph nodal status was not described.[18] Among these studies,[18–20,22] we consider that an association between amplification or overexpression of c-*erb*B-2 and poor patient prognosis or tumor recurrence might have been demonstrated if cases with positive lymph nodal metastasis had been examined.

Slamon *et al.* described that fixation and embedding of tissue frequently decreases or totally destroys the reactivity of many antigens.[7] However, in the present study, the copy number of the c-*erb*B-2 gene and expression of the c-*erb*B-2 protein examined by immunohistochemistry were completely coincident even after storage of the tissue blocks for more than 10 years. Thus, immunohistochemical study of c-*erb*B-2 expression using a polyclonal antibody is probably a useful tool for detecting breast carcinoma patients with poor prognosis by both prospective and retrospective analyses. However, one should take into account not only the status of the c-*erb*B-2 gene or protein, but also other prognostic factors, e.g., lymph nodal status, tumor size, hormone receptor status, menstrual status and surgical therapy. It seems to be important to study multiple data on prognostic factors using multivariate analysis such as Cox's proportional hazards linear model in order to predict the risk of recurrence or metastatic spread in each patient after initial surgery.

Other than amplification of the gene itself, a mechanism of activation of the c-*erb*B-2 gene seemed to be present because a proportion of cases were shown to overexpress the c-*erb*B-2 gene without amplification. However, the major mechanism of c-*erb*B-2 gene activation in breast carcinoma is considered to be amplification, because in this study the majority of positive cases, especially those showing strongly positive immunostaining, were associated with amplification of the c-*erb*B-2 gene, although the possibility remains that the c-*erb*B-2 gene is activated by point-mutation, as shown in mammary carcinoma in rats,[23] and that such activation is difficult to detect immunohistochemically.

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