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Construction of Immunoradiometric Assay for Circulating c-*erb*B-2 Protooncogene Product in Advanced Breast Cancer Patients

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The human c-*erb*B-2 protooncogene product (*erb*B-2 protein) is a 185 kilodalton glycoprotein closely related to epidermal growth factor receptor protein. In this study, we measured the concentration of circulating *erb*B-2 protein in cancer patients by means of a new immunoradiometric assay (IRMA). Two monoclonal antibodies (MoAbs), SV2-61γ and 6G10, recognize *erb*B-2 protein but bind to separate epitopes. SV2-61γ was used as an immunoadsorbent and 6G10 as an ^{125}I-labeled probe. A serum was considered positive for *erb*B-2 protein if the percent binding exceeded the mean of the normal group by more than 3 standard deviations. Eleven of 21 patients with advanced breast cancer and 1 of 15 with advanced gastric cancer were positive. Serum *erb*B-2 protein levels correlated well with the therapy and the status of the patients with breast cancer. On the contrary, all patients with advanced colon, ovarian, or pancreatic cancers, showed levels below the cut-off value. These results suggest that circulating *erb*B-2 protein can be measured using the newly constructed IRMA. Since c-*erb*B-2 protooncogene amplification and overexpression are accepted as a good marker of aggressiveness, relapsing potency, and poor prognosis, this IRMA should be a promising tool with which to help manage breast cancer patients.

Key words: Tumor marker — c-*erb*B-2 product — Immunoradiometric assay — Breast cancer

The c-*erb*B-2 protooncogene encodes a protein that is similar to the epidermal growth factor (EGF) receptor but is distinct from it.[1)] Recently we reported that the c-*erb*B-2 protooncogene product (*erb*B-2 protein), overexpressed on the surface membranes of various adenocarcinomas, is a useful target in immunoscintigraphy using monoclonal antibodies (MoAbs).[2)] This protein is reportedly expressed in various adenocarcinomas including breast, gastric, and colon cancers,[3)] whereas EGF receptors are expressed on squamous cell carcinomas and gliomas as well as adenocarcinomas.[4-6)] Amplification and overexpression of the c-*erb*B-2 gene correlate with tumor aggressiveness and unfavorable clinical outcome, especially in patients with breast or gastric cancer.[7-11)] In addition, this protein is detectable in the sera of breast cancer patients.[3, 12)] In this study, we constructed an immunoradiometric assay (IRMA) to determine whether circulating *erb*B-2 protein is detectable in the sera of patients with breast, gastric, colon, ovarian and pancreatic cancers.

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MATERIALS AND METHODS

Monoclonal antibodies MoAbs SV2-61γ and 6G10 (Nichirei, Tokyo) recognize the extracellular domains of *erb*B-2 protein, and bind separate epitopes. SV2-61γ (IgG1) was generated by immunizing mice with SV11 cells in which the c-*erb*B-2 complementary DNA was expressed.[13)] The MoAb 6G10 (IgG2a) was obtained by immunizing mice with recombinant c-*erb*B-2 gene-transfected A4 cells.[3)]

Standard antigens Standard antigens were obtained by solubilizing the cell membrane of A4 (c-*erb*B-2 transfected NIH3T3) cells cultured in Eagle's minimum essential medium (Nissui Pharmaceutical Co., Tokyo) supplemented with 10% fetal calf serum and 0.03% L-glutamine. The cells were transplanted subcutaneously into a BALB/c-*nu/nu* mouse, then 2 weeks later, the A4 xenograft was removed. Cells were obtained by passing tumor specimens through a stainless steel mesh and were suspended in phosphate-buffered saline (PBS). After addition of 4 ml of 0.5% Triton X-100 in PBS, 1×10^8 cells were incubated on ice for 1 h. Nuclei and debris were removed by centrifugation (10,000*g*, 10 min). The supernatant was serially diluted with dilution buffer (Daiichi Radioisotope Laboratories Ltd., Tokyo) and the antigen

level of the supernatant diluted to 1/8 was arbitrarily defined as 500 U/ml.

Preparation of beads Polystyrene beads (7 mm in diameter) were coated with MoAb SV2-61γ in 1 m*M* phosphate buffer, pH 6.4 containing 5% MoAb at 4°C for 20 h. The beads were washed three times with distilled water, and non-specific binding was blocked by processing in 50 m*M* PBS containing 0.5% bovine serum albumin (BSA).

Radio-iodination of monoclonal antibody MoAb 6G10 was radio-iodinated using the chloramine-T method. Purified antibody (20 μg) in 0.3 *M* phosphate buffer, pH 7.5 and ^{125}I (0.4 mCi) for protein labeling (Amersham International plc, Buckinghamshire, UK) were mixed with 2.5 μg of chloramine-T (Nakarai Chemicals, Kyoto). After a 5 min incubation, ^{125}I-labeled MoAb was separated from free ^{125}I by passage through a Sephadex G-25 gel column. The specific activity was 444 MBq (12 mCi)/mg.

Sera Sera were obtained between 1989 and 1990 in Kyoto University Hospital from patients with breast cancer (n=21), ovarian cancer (n=11), colon cancer (n=15), gastric cancer (n=15), and pancreatic cancer (n=10). Serum samples were stored at −20°C until use. All cancer patients underwent treatment in Kyoto University Hospital, and diagnoses were based upon surgical and/or histological findings. Clinical examinations, laboratory tests, and radiological findings demonstrated that all of them were in Stage IV or the recurrent stage, at the time of serum sampling. Sera were also taken from 20 normal adults; 10 males and 10 females.

Immunoradiometric assay An IRMA was constructed using SV2-61γ as the immunoadsorbent and 6G10 as the ^{125}I-labeled probe. Radio-iodinated 6G10 was diluted with tracer dilution buffer (Daiichi Radioisotope Laboratories, Ltd.) at a specific activity of 60,000 cpm/100 μl. Standard antigens or serum samples (50 μl) were incubated with 100 μl of ^{125}I-labeled 6G10 and SV2-61γ-coated beads for 6 h at room temperature. The beads were washed three times with distilled water, and the radioactivity bound to the beads was measured using a well-gamma counter. Bound radioactivity was converted to *erb*B-2 protein U/ml by reference to a standard curve.

CA15-3 assay In all the patients with breast cancer, the serum CA15-3[14, 15] levels were determined using a commercially available kit (Centocor, Malvern, PA) with a cut-off value of 28 U/ml. The procedure of the assay was as described by the manufacturer. CA15-3 levels were compared with those of *erb*B-2 protein.

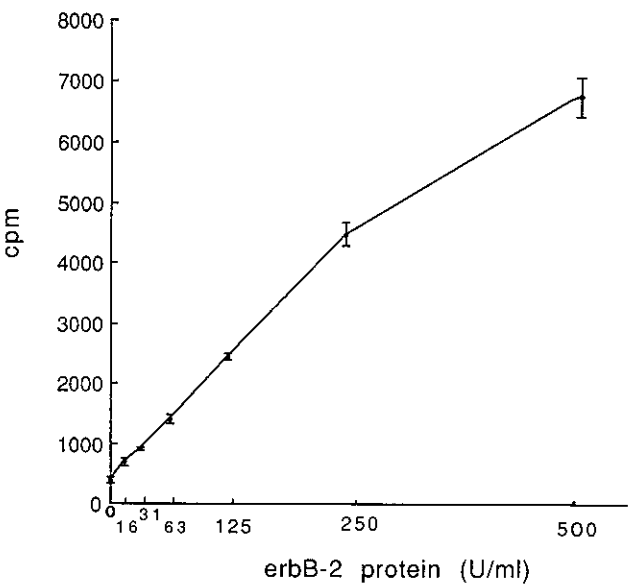

Fig. 1. Standard curve. Bars indicate the standard deviations of five different assays. cpm: radioactivity of the beads.

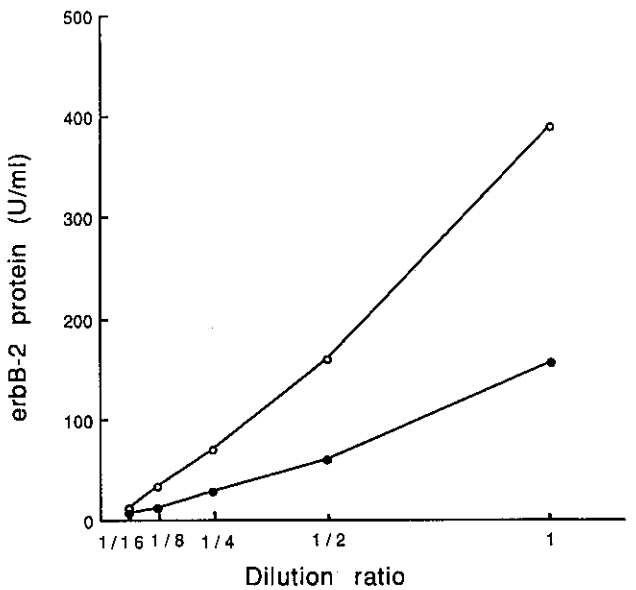

Fig. 2. Two samples of *erb*B-2 protein-positive serum were diluted and measured by the IRMA.

RESULTS

The standard curve of the assay is shown in Fig. 1. The prozone phenomenon was observed with the less diluted supernatant of the standard antigens. Two of the serum samples were diluted with dilution buffer in geometrical series, and the *erb*B-2 protein levels of diluted samples are illustrated in Fig. 2. Both of the plots showed good

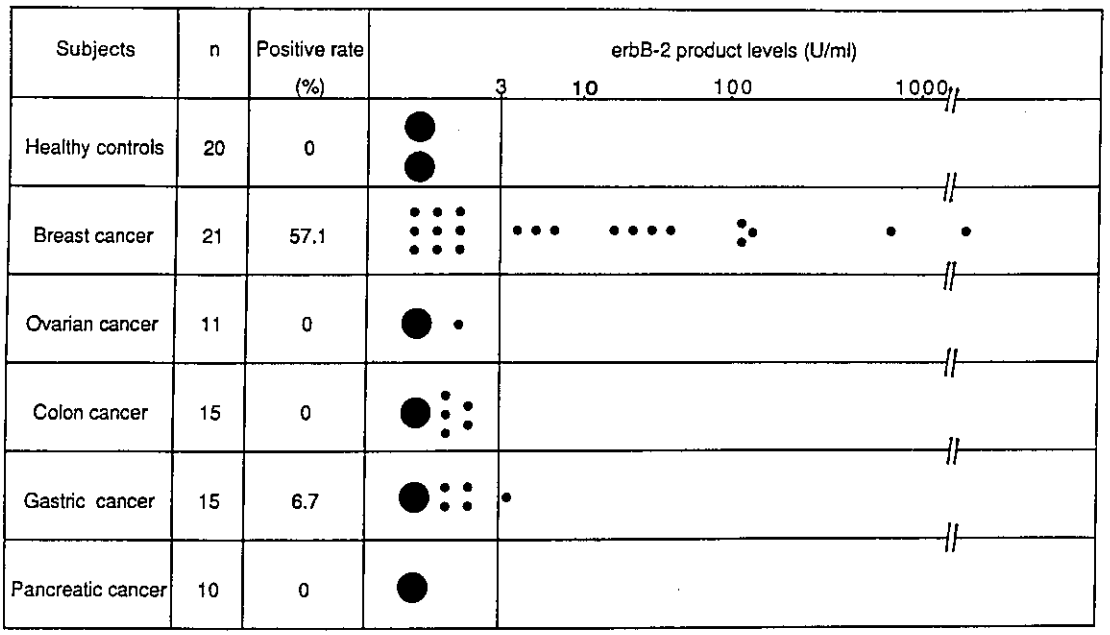

Fig. 3. Serum c-*erb*B-2 product levels in healthy controls and adenocarcinoma patients. One large symbol (●) indicates ten patients.

Table I. Relationship between *erb*B-2 Protein and CA15-3 Levels in the 21 Advanced Breast Cancer Patients

*erb*B-2 protein	No. of patients (%)	
	CA15-3	
	Positive	Negative
Positive	11 (52.4)	1 (4.8)
Negative	4 (19.0)	5 (23.8)

linearity from 1 to 1/16 dilution. In the 20 healthy controls, the *erb*B-2 protein concentrations ranged from 0 to 2.6 U/ml. The mean and the standard deviation were 0.4 and 0.7, respectively, and there was no difference between males and females. The cut-off value was 3 U/ml, determined as a mean plus 3 standard deviations. All the healthy controls showed levels below the cut-off value (Fig. 3). Twelve of 21 patients (57.1%) with advanced breast cancer were positive. On the contrary, all 15 patients with colon cancer, all 11 patients with ovarian cancer, all 10 patients with pancreatic cancer, and 14 of 15 patients with gastric cancer had *erb*B-2 protein levels below the cut-off value of 3 U/ml.

Fifteen of 21 patients (71.4%) had positive CA15-3 levels with a cut-off level of 28 U/ml. The patients were divided into four groups on the basis of *erb*B-2 protein and CA15-3 levels (Table I). The correlation coefficient between *erb*B-2 protein and CA15-3 was 0.425.

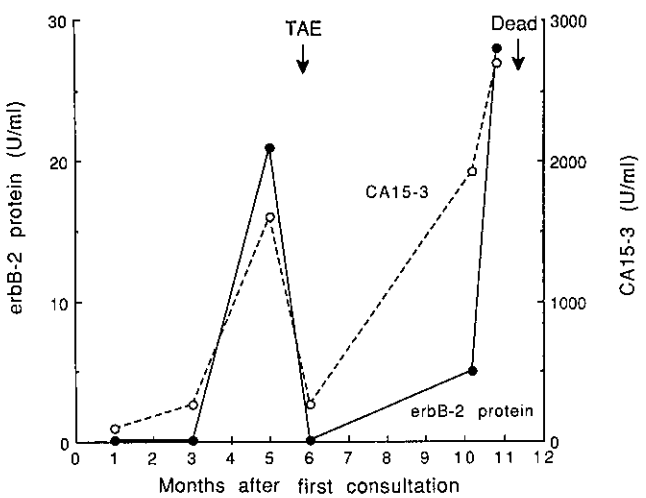

Fig. 4. Patient A: 53-year-old female with breast cancer showing liver, lung, and bone metastases. TAE: transcatheteral arterial embolization of metastatic liver lesions. Serum *erb*B-2 protein levels (●); CA15-3 levels (○).

Serum samples were serially taken from 2 patients (A and B) with breast cancer, and the changes of serum *erb*B-2 protein levels and CA15-3 levels were determined.

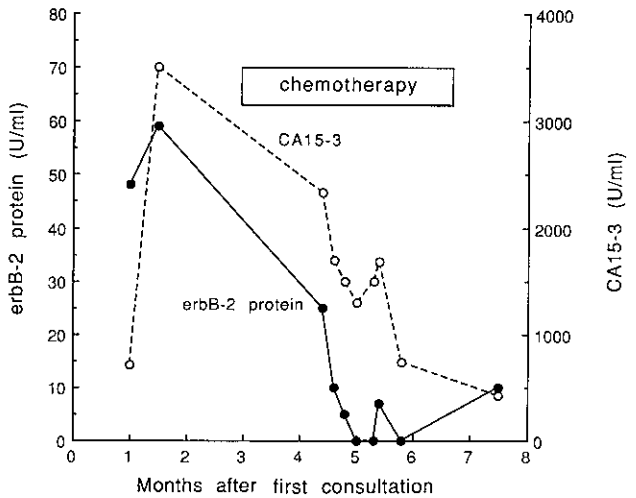

Fig. 5. Patient B: 52-year-old female breast cancer patient with bone and bone marrow metastases. Chemotherapy was continued for 3 months in hospital. Serum *erb*B-2 protein levels (●); CA15-3 levels (○).

Patient A, a 53-year-old female, who had undergone left mastectomy 2 years previously, had multiple liver metastases, bone metastases, lung lymphangitis carcinomatosa and ascites. Liver metastases were treated by transcatheter arterial chemoinfusion and embolization, after which the serum *erb*B-2 protein level decreased. However, the *erb*B-2 protein levels increased again with the extension of metastatic lesions. She expired 11 months after the diagnosis of recurrence (Fig. 4). Patient B, a 52-year-old female with left breast cancer, had multiple bone, bone marrow and brain metastases at the time of the first consultation, and mastectomy was not performed. During admission, she was treated by chemotherapy using fluorouracil, cyclophosphamide and tamoxifen citrate. Metastatic lesions improved and the serum *erb*B-2 protein level decreased (Fig. 5).

In both patients, serum *erb*B-2 protein levels determined by our IRMA changed in parallel with serum CA15-3 in response to the therapy and the status of patients.

DISCUSSION

The protooncogene c-*erb*B-2 encodes a 185 kilodalton transmembrane tyrosine kinase, which is similar to EGF receptor, although EGF does not bind to or activate *erb*B-2 protein. Recently heregulin (HRG) proteins interacting specifically with *erb*B-2 protein have been identified as the probable natural ligands for *erb*B-2 protein.[16]

Overexpression of the *erb*B-2 protein, generally as a consequence of gene amplification, has been reported in human breast, gastric, ovarian, colon, pancreatic, and urinary bladder cancers. Some clinical studies have disclosed association of c-*erb*B-2 gene amplification and aggressiveness, relapsing potency and poor prognosis of these cancers.[7–11, 17–20]

In this study, we detected and quantified *erb*B-2 protein in sera from patients with adenocarcinomas. Patients with breast cancer had high concentrations and positive rates compared with other adenocarcinoma patients. According to some immunohistochemical reports, about 30% of breast cancer patients had *erb*B-2 protein.[21, 22] An immunohistochemical study has shown that 28 (14.3%) of 196 advanced gastric cancer cases were positively stained for *erb*B-2 protein.[11] In our study, 57.1 and 6.7% of breast and gastric cancer patients, respectively, had positive serum *erb*B-2 protein. Our breast cancer patients demonstrated a higher positive rate than those studied immunohistochemically and reported elsewhere. This may be because all our patients were in the advanced stages. Yu *et al.* reported that 45.7% of the postoperative recurrent breast cancer patients were positive for serum *erb*B-2 protein.[12] Their positive rate may be considered equivalent to that of our advanced cancer patients.

CA15-3, a tumor marker detectable by two monoclonal antibodies 115D8 and DF3, has been used for the diagnosis and follow-up of breast cancer patients. The positive rate of CA15-3 in advanced stages is reported to be 70%.[23, 24] In our study, 57.1% of advanced breast cancer patients had positive serum *erb*B-2 protein levels, while 71.4% of them showed positive CA15-3. Furthermore, *erb*B-2 protein showed only a weak correlation with CA15-3. One patient showed positive *erb*B-2 protein and negative CA15-3, and 4 patients were negative for *erb*B-2 protein and positive for CA15-3. Thus, *erb*B-2 protein is not only biochemically a completely distinct substance from the antigen determined with CA15-3 assay, but it may be considered as a different tumor marker from CA15-3 in monitoring breast cancer patients. Further study with more patients must be undertaken to estimate the usefulness of this *erb*B-2 protein assay in comparison with CA15-3.

Gene amplification and overexpression of c-*erb*B-2 have clinical prognostic significance for patients with c-*erb*B-2-positive tumors. Thus, gene analyzing and immunohistochemical tests are essential in defining an individual prognosis. However, these tests need tumor specimens, require a lot of time and labor, and cannot deal with many samples in a short time. Compared with them, IRMA, a much simpler method, has several advantages; patient's serum can be obtained easily and repeatedly, many samples are measured at the same time, and IRMA

can quantify them numerically. In addition, our construction of IRMA measuring an oncogene product suggests that other oncogene products can be determined with IRMA, and such serodiagnoses may supplement or replace gene analyzing and immunohistochemical tests for some clinical purposes. Although additional studies, including patients in the earlier stages, will be necessary to define the predictive value of serum *erb*B-2 protein, our IRMA for *erb*B-2 protein seems to be a useful tool with which to monitor breast cancer patients.

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