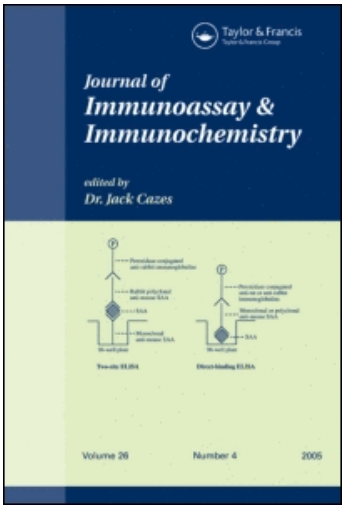

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ELISA FOR QUANTITATION OF SERUM C-erbB-2 ONCOPROTEIN IN BREAST CANCER PATIENTS

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ABSTRACT

C-erbB-2 is a protooncogene that is overexpressed in various cancers, either due to its amplification and/or increased transcription, and has been associated with more aggressive disease and a poor clinical prognosis in 20–30% of patients with breast cancer. Besides the prognostic factors such as tumor size, tumor grade, lymph node status, etc., which are significant in the management of breast cancer, C-erbB-2 level might also serve as an additional factor. Immunohistochemistry is the most frequently used method to study the expression of C-erbB-2 in breast cancer. We have generated a panel of monoclonal antibodies against C-erbB-2 oncoprotein with a view to evaluate their application for the diagnosis and therapy of breast cancer. In the present study, a simple, quantitative sandwich ELISA has been developed that

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uses two monoclonal antibodies directed against the extracellular domain of C-erbB-2 product, designated as CIBCgp185 as the capture antibody and CIBCHER-2 as the detector antibody. C-erbB-2 protein, isolated from BT474 cells, a human breast carcinoma cell line with high expression of C-erbB-2 and purified by Concanavalin A-Sepharose 4B affinity chromatography and HPLC has been used to develop the ELISA procedure. Sera samples of 150 healthy women donors and of 300 breast cancer patients with different histological types of malignancies have been analysed. The control women had serum C-erbB-2 in the range of 4.0–13.2 ng/mL, whereas the 300 breast cancer patients studied had a range of 4.8–75.2 ng/mL with a cut off value of 13.8 ng/mL. Our study showed that 18.6% of breast cancer patients had elevated levels of circulating C-erbB-2. These results might suggest that the serum C-erbB-2 level can be used as a potential tumor marker for breast cancer and that the Sandwich ELISA procedure might serve as an excellent alternative to immunohistochemistry in the near future.

Key Words: C-erbB-2; ELISA; Breast cancer

INTRODUCTION

The product of C-erbB-2 Protooncogene is a 185KD transmembrane glycoprotein possessing tyrosine kinase activity.[1] Amplification of this gene and overexpression of the product has been correlated with poor prognosis in patients with breast cancer, which constitutes the second most common malignancy among South Indian female population.[2,3] Many reports have shown that the C-erbB-2 protein is overexpressed in several tumors of epithelial origin including breast, lung, hepatocellular, prostate, pancreas, colon, ovarian, cervical, and bladder. Antibodies directed to the external domain of this protein have proved useful not only for immunohistochemistry, but also for the diagnosis and therapy of patients with overexpression of C-erbB-2.[4,5]

Assay of C-erbB-2 overexpression has gained so much clinical importance in the past few years, because of the U.S. Food and Drug Administration (FDA) approval of Trastuzumab (Herceptin), a humanised monoclonal anti-C-erbB-2 antibody for the treatment of patients with metastatic breast cancer.[6] Anti C-erbB-2 monoclonal antibody therapy with

Trastuzumab is approved for the treatment of metastatic breast cancer in the USA and it is in this context where there is an absolute necessity for an accurate C-erbB-2 assessment before appropriate implementation of therapy. The C-erbB-2 receptor analysis can be performed by immunohistochemical assay (IHC) to visualize the level of expression or by quantitation in tissue and cell culture extracts by enzyme linked immuno sorbent assay (ELISA).[7,8]

With a view to develop such an immunoassay procedure, we have generated a panel of monoclonal antibodies against the extracellular domain of C-erbB-2 oncoprotein. Two of these monoclonal antibodies, designated as CIBCgp185 and CIBCHER-2, have been extensively characterized and their specificity evaluated by various immunological assays. Immunohistochemical assay of frozen tissue sections of breast tumors of different histological types and of other types of cancers using monoclonal antibody CIBCgp185 against C-erbB-2 has revealed that 28% of breast cancers overexpress C-erbB-2.[9]

We describe here the development and clinical application of an enzyme immunoassay using these two monoclonal antibodies for quantitation of circulating levels of serum C-erbB-2 in breast cancer patients.

EXPERIMENTAL

Materials

All fine chemicals, such as acrylamide, TEMED, ammonium persulphate, 2-mercaptoethanol, 3,3′,5,5′ tetramethyl benzidine, Concanavalin A-Sepharose 4B, and Triton × 100, were purchased from the Sigma Chemical Company, USA. Peroxidase conjugated rabbit antirat immunoglobulin was purchased from Dako, Denmark. Other fine chemicals were obtained from E. Merck and BioRad. Foetal Bovine Serum (FBS), Dulbecco's Modified Eagle's Medium (DMEM) and glutamine were from Invitrogen (India) Ltd (Formerly GIBCO BRL). 96 Well Falcon ELISA plates were supplied by Becton Dickinson, USA. ^{125}I was purchased from BRIT, BARC, Mumbai.

Breast Cancer Cell Line and Monoclonal Antibodies

Breast carcinoma cell line BT474, which overexpresses C-erbB-2 and control antibody ICR12[10] against C-erbB-2, were a gift from the late Dr. C. J. Dean, Institute of Cancer Research, UK. Monoclonal antibodies

CIBCgp185, a mouse MAb and CIBCHER-2, a rat MAb recognizing different epitopes in the extracellular domain of C-erbB-2 oncoprotein, were generated in this laboratory.[11] Briefly, mouse monoclonal antibodies against C-erbB-2 oncoprotein were generated by hybridization of immune spleen cells of BALB/c mouse immunized with BT474 cell lysate and Sp2/0 myeloma cells using 50% PEG as the fusing agent, according to the method of Galfre and Milstein.[12]

Blood Samples

Blood samples of normal healthy female donors were collected from the Cancer Institute blood bank and of cancer patients from the out patient department at the time of initial presentation; sera were separated, aliquoted, and stored at -70°C until used for the assay. Blood from two male patients with breast cancer was also collected. Informed consent was obtained from each patient before blood collection.

Preparation of BT474 Cell Lysate

C-erbB-2 protein-rich membrane preparation for enzyme immunoassay was obtained from lysates of BT474 cells. Cell lysates were prepared by adding 1 mL of lysis buffer that consisted of 50 mM Tris–HCl pH 7.5, 1% Triton $\times$ 100, 150 mM NaCl, 1 mM phenyl methyl sulfonyl fluoride (PMSF) to BT474 cells and incubated for 10 min. Extracts were clarified by centrifugation at 10 000 *g* for 10 min to obtain a clear supernatant, which was again centrifuged at 40 000 *g* for 20 min to obtain the cell membrane. All procedures were performed on ice in a 4°C cold room. Further purification was achieved by performing Concanavalin A-Sepharose 4B affinity chromatography, as detailed earlier.[13] The fractions were monitored at 280 nm and the C-erbB-2 protein-rich fractions were pooled and dialysed against deionized water. Further purification was carried out by HPLC, using an E. Merck HEMA gel filtration column equilibrated with Tris–HCl buffer and the C-erbB-2 protein extracted from the column using the same buffer at a flow rate of 3 mL/min. After dialysis, the pooled fractions were lyophilized and used for the study. The protein concentration of the pure C-erbB-2 membrane extract was determined by the method of Lowry et al.[14] The homogeneity of the purified C-erbB-2 protein was analysed by SDS-PAGE using 7.5% gel according to the method of Laemmli.[15]

Competitive Binding Assay

The binding of these two antibodies to different epitopes on the extracellular domain of C-erbB-2 protein was assessed by competitive binding assay. This assay was performed using BT474 cells as target. The cells were collected from a confluent flask and washed in PBS containing 2% BSA. The test was carried out in triplicate. To 1.0 mL cell suspension in separate vials, 100 μL ^{125}I-CIBCgp185 (50 000 cpm) and 50 μL of 2% BSA were added. This will serve as control, to determine maximum binding. To the other set of 3 vials with 1.0 mL cell suspension, 100 μL of ^{125}I-CIBCgp185 and 50 μL of monoclonal antibody CIBCHER-2 (50 μg/mL) were added. After 45 min incubation on ice, the cells were washed four times with PBS/BSA, lysed, and the bound radioactivity determined in an LKB Rack Gamma counter.

Sandwich ELISA Protocol

A sandwich immunoassay was developed using MAb CIBCgp185 as capture antibody and MAb CIBCHER-2 as detector antibody. Hundred microlitres of the capture antibody (5 μg/mL) was added to each well of the microtitre Falcon plates and the plates kept in the refrigerator overnight for absorption of the antibody .The plates were washed in Tween 20/PBS and then in PBS thrice. 100 μL of 2% BSA in PBS was added per well and incubated for 1h at 37°C to block non specific binding. The test was carried out in duplicate. Hundred microlitres of the serum samples (1 : 50 in PBS containing 1% BSA and 10% NMS) was added to the wells and incubated for 3 h at 37°C. After washing in Tween 20/PBS and then in PBS thrice, the bound serum C-erbB-2 was detected by incubating with 100 μL of MAb CIBCHER-2 (5 μg/mL) for 1 h, and then for 30 min with 100 μL of peroxidase conjugated rabbit antirat immunoglobulin (1 : 1000 in 2% BSA/PBS), followed by addition of 100 μL of TMB substrate (tetramethylbenzidine in phosphate buffer and 0.03% hydrogen peroxide). After incubation for 10 min in the dark, 50 μL of 2 M sulphuric acid was added to each well to arrest the reaction and the plates were scanned at 450 nm in a Biotek ELISA reader.

Both positive and negative controls were included in the test. As negative control, 100 μL of Normal mouse serum (1 : 100 in PBS) was used instead of the detector antibody and anti-C-erbB-2 antibody ICR12 as the positive control. A calibration curve was constructed by incubating different concentrations of pure C-erbB-2 protein (0–35 ng/mL) in the ELISA procedure.

RESULTS

Based on the reactivity of the monoclonals we had generated against the C-erbB-2 antigen, we selected the MAb CIBCgp185 as the capture antibody for the sandwich ELISA test.

C-erbB-2 membrane protein, extracted from BT474 cells, and purified by Concanavalin A-Sepharose 4B affinity chromatography and HPLC, had a protein concentration of 4 mg/mL. When subjected to SDS-PAGE on 7.5% gel, it was found to be a homogenous 185 KD protein (Fig. 1).

In competitive binding assay, antibody CIBCgp185 was bound to a unique epitope and its binding was not inhibited by CIBCHER-2. This clearly indicated that the two antibodies recognize two different epitopes on the extracellular domain of C-erbB-2.

The calibration curve constructed using different concentrations of purified C-erbB-2 protein (0–35 ng/mL) and the MAb CIBCgp185 as the capture antibody and CIBCHER-2 as the detector antibody, gave a straight line and was used for interpolation of C-erbB-2 values of sera samples (Fig. 2).

The median age of the breast carcinoma patients was 52 years (range 22–71) and that of the control group was 36 years (range 26–53 years). The serum C-erbB-2 levels for 150 healthy women donors were in the range of 4.0–13.2 ng/mL, with a mean S.D. of 10.2 ± 1.8 ng/mL, with a cut off value of 13.8 ng/mL (defined as 2 standard deviations above the mean of the controls). The 300 breast cancer sera studied had C-erbB-2

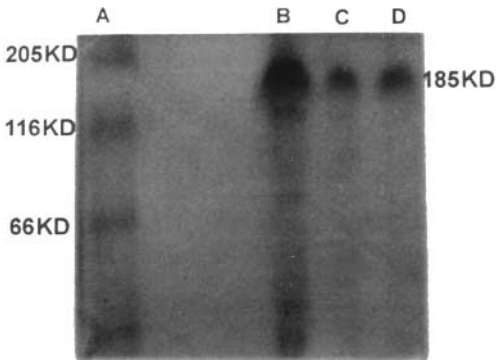

Figure 1. SDS-PAGE analysis of purified C-erbB-2 protein using 7.5% gel: Indicates a homogenous 185 KD protein. Lane A: Molecular weight markers. Lane B: Partially purified C-erbB-2 protein. Lanes C and D: Purified C-erbB-2 protein.

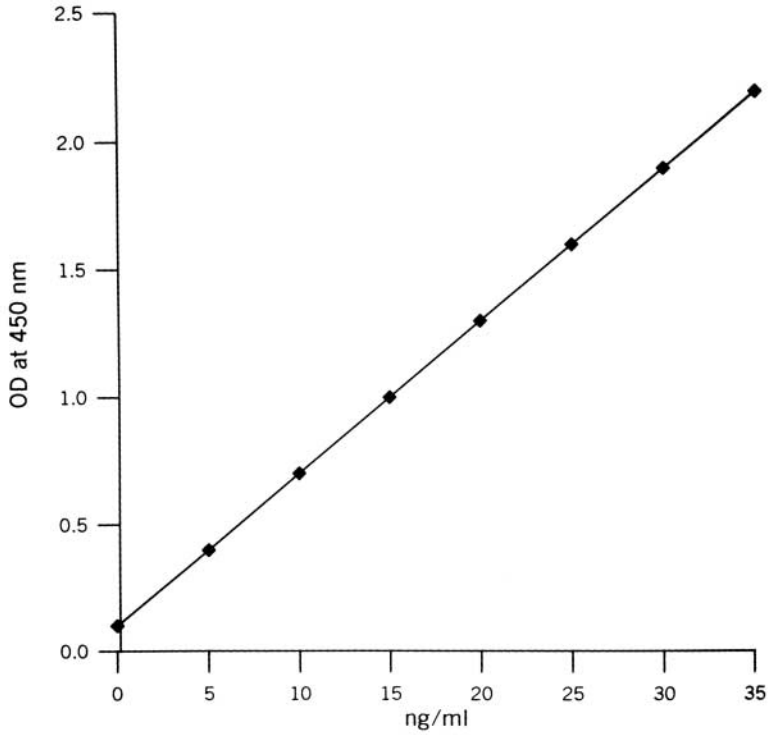

Figure 2. Calibration curve for serum C-erbB-2.

levels in the range of 4.8–75.2 ng/mL (Fig. 3) and it was found that 56 patients with breast carcinoma (18.6%) showed elevated C-erbB-2 levels above a cut off value of 13.8 ng/mL. Two male patients with breast cancer included in the study had normal serum C-erbB-2 levels.

DISCUSSION

A wide range of assay methods has been used and they are being developed to analyse tumor C-erbB-2 status, although some of them are only suitable for research purposes rather than routine screening of patients.[16] Various target molecules related to C-erbB-2 amplification/overexpression, such as DNA, mRNA, and receptor protein, can be used in different assays. Each technique has advantages and disadvantages which need to be weighed against each other in any assessment of the

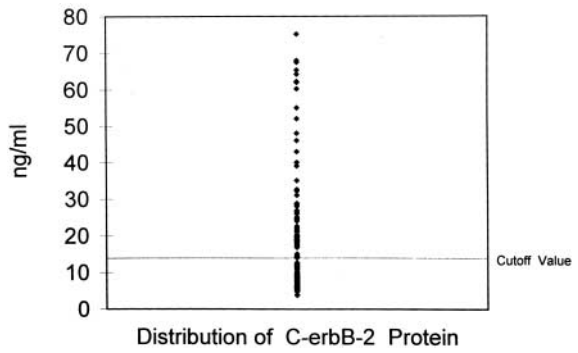

Figure 3. Distribution of C-erbB-2 protein in sera of 300 breast cancer patients.

most appropriate technique for routine laboratory assessment of C-erbB-2 status.[17] For assessment of C-erbB-2 amplification, fluorescent in situ hybridisation (FISH) is the sensitive and standard assay. However, very few laboratories are set up to assay C-erbB-2 status using FISH and it is also relatively time consuming, expensive, and complicated to perform. Immunohistochemical staining of cancer tissue is the most widely used and is a relatively inexpensive method, at present, to evaluate the overexpression of C-erbB-2.[18] However, immunohistochemistry (IHC) has difficulty in the quantification of the level of C-erbB-2 overexpression. C-erbB-2 positivity in breast cancer patients, as evaluated by IHC, varied from 20–30%. C-erbB-2 is a well characterized oncogene that has been directly implicated in the progression of breast cancer and poor survival of patients. Considerable evidence from the literature points out the fact that a soluble fragment (extracellular domain) of the C-erbB-2 oncogene product may be released from the cell surface, become detectable in the serum of patients with breast cancer, and have been found to be useful in detecting the presence of metastatic tumors.[19–22] The mechanism of release of soluble C-erbB-2 product remains controversial. An enzyme linked immunosorbent assay (ELISA) on serum would be ideal, as it is convenient and economical and may be applied in patients for whom no representative archival tissue materials are available for immunocytochemical assay.

Herceptin is a humanised genetically engineered antibody which has been evaluated for its diagnostic and therapeutic potential. Herceptin therapy for treatment of tumors with overexpression of C-erbB-2 oncoprotein has promising application in the management of breast cancers which do not respond to routine modalities of therapy such as radiotherapy and chemotherapy.[23,24] It is a relatively simple technique and is well suited

for automation. Determination of serum C-erbB-2 antigen may prove useful in selecting patients for herceptin treatment towards the C-erbB-2 receptor and in the follow-up of patients receiving such therapy, or as a predictor of response to chemotherapy, endocrine therapy or radiation therapy.[25–27]

Recognising the overall oncological importance of C-erbB-2 oncogene and with a view to study the expression of C-erbB-2 in breast cancer, the two monoclonal antibodies CIBCgp185 and CIBCHER-2 generated in our laboratory against the extra cellular domain of C-erbB-2 oncoprotein were used in this assay. By immunohistochemical assay, these monoclonal antibodies revealed that 28% of the breast cancers had high expression of C-erbB-2. These results are in good correlation with those reported by other workers.[28] The elevated expression of C-erbB-2 in these tumors might also be reflected in the circulating level of C-erbB-2 in these patients. C-erbB-2 levels in the serum of patients with breast carcinoma were detected by ELISA using monoclonal antibodies which recognize different epitopes on the extracellular domain of C-erbB-2 receptor.

This current study allowed easy quantification of C-erbB-2 level in serum. Serum C-erbB-2 assay for 150 healthy control women and 300 breast cancer patients by sandwich ELISA revealed that 18.6% of these patients had elevated levels with a cut off value of 13.8 ng/mL. The values were interpolated from a calibration curve prepared using C-erbB-2 protein isolated from BT474 cancer cell line with overexpression of C-erbB-2 and purified by Concanavalin A-Sepharose 4B affinity chromatography and HPLC.

The percentage coefficient of variation between assays determined by performing 4 separate assays using 6 samples of different concentrations of C-erbB-2 protein was not very significant.

The majority of tumor markers in clinical use today show a substantial degree of correlation between the disease course and the level of tumor marker in the serum.[29] The same is the case with C-erbB-2.[30] The assessment of C-erbB-2 status in breast carcinomas is of great importance for clinical management, since it provides valuable prognostic and predictive information, particularly when used along with routine breast cancer markers.[31,32] The value of this marker is hampered by the lack of standardization of the various methodologies and scoring systems for determining the C-erbB-2 status of a given tumor. At present, there is no consensus on the most reliable method to determine C-erbB-2 status as a prognostic marker; as a predictor of response to chemotherapy, endocrine therapy or radiation therapy, or as a way to assign patients to receive herceptin treatment. The current study suggests that Serum C-erbB-2 may prove to be a clinically important tumor marker in monitoring of breast cancer patients with overexpression of C-erbB-2. The serum C-erbB-2 ELISA is

a non-invasive procedure and, if the results correlate with IHC, it might serve as an appropriate alternative to immunohistochemistry.

All these findings might lead to the conclusion that our MAbs against C-erbB-2 have promising future application for serological determination of C-erbB-2 by the sandwich ELISA technique. This immunoassay procedure has to be further evaluated by performing the test on a large number of samples and controls to establish its clinical usefulness. There is also a need to focus on standardizing methodologies to exactly quantitate the level of C-erbB-2 in breast cancer patients and to apply common interpretation criteria to evaluate inter-laboratory variations.

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