Amplified and Overexpressed Epidermal Growth Factor Receptor Gene in Uncultured Primary Human Breast Carcinoma

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ABSTRACT

We analyzed the epidermal growth factor receptor gene using a complementary DNA probe of the epidermal growth factor receptor gene in 21 uncultured primary breast carcinomas and found that the gene was amplified in three of these tumors. We further demonstrated by immunohistochemistry using a monoclonal antibody to the epidermal growth factor receptor that the receptor protein product of this gene was overexpressed and displayed elevated kinase activity. Our data indicate that one of the molecular mechanisms for overexpression of epidermal growth factor receptor in human breast cancer is epidermal growth factor receptor gene amplification without rearrangement in a subset of tumors.

INTRODUCTION

EGF-r is a M, 170,000 membrane protein exhibiting an extracellular binding domain that serves to bind the ligand, EGF or transforming growth factor α, a transmembrane region, and an intracellular domain facing the cytoplasm exhibiting the tyrosine kinase function (1). EGF has been shown to stimulate the growth of human breast cancer cells (2), and human breast cancer cell lines secrete a factor with transforming activity (3). Amplification of EGF-r and of an EGF-r-related gene has been reported in two cell lines derived from human breast tumor (4, 5). Although EGF-r is detected in nearly ½ of uncultured human breast tumors by a radioreceptor binding assay or by immunohistochemical studies (6–9), an extensive analysis of the EGF-r gene for a possible mechanism to explain this overexpression has not been reported in these tumors.

To determine the EGF-r gene structure in uncultured human breast tumors, we analyzed 45 breast tissues (21 carcinomas, 20 adjacent nontumorous breast tissues, 3 adenomas, 1 gynecomastia) by Southern blot analysis and/or DNA dot blot analysis. In a limited number of these samples, we studied EGF-r expression and kinase activity.

MATERIALS AND METHODS

Procurement of Tissue. Forty-five diagnostic specimens were obtained at the University of Texas, M. D. Anderson Hospital and Tumor Institute (Table 1) and reviewed by a member of the Department of Pathology. Table 1 details the clinical, pathological, and biochemical features of the 21 patients from whom the breast carcinomas were removed. Breast tissue specimens were obtained immediately after surgery and stored at −70°C or processed immediately by placing them in a mortar containing liquid nitrogen. The frozen tissue was then pulverized with a pestle. High-molecular-weight DNA was prepared from the pulverized tissue powder (10).

Blot Hybridizations. All DNAs were digested with 2 or 3 restriction endonucleases (EcoRI, HindIII, and PstI), size fractionated in a 0.8% agarose gel, denatured, neutralized, and transferred to a nylon membrane as described (11). The filters were hybridized for 48 h at 42°C, washed in 0.1 × standard saline citrate with 0.1% SDS at 60°C for 60 min, and exposed to X-ray film at −70°C. Sequential hybridizations were carried out on the same filter after boiling them for 10 min to remove the previous probe.

To estimate the gene copy number, DNA (64 μg) was serially diluted, denatured, neutralized, and dot blotted onto a nylon filter. Hybridization, rehybridizations, and washings were carried out as described above.

Immunoperoxidase Staining. Frozen section slides were prepared, and anti-EGF-r antibody (R1) (1:100 Amersham International, Amersham, United Kingdom) was incubated for 1 h on these tissue sections. Antibody reactivity was determined using the Vectastain ABC (peroxidase) anti-mouse IgG procedure (Vector Laboratories, Burlingame, CA), with diaminobenzidine (1 mg/ml in Tris-HCl, pH 7.2) as substrate as described (12). The sections were counterstained with hematoxylin.

Immunoperoxidase Kinase Assay. For kinase assays, tissue was lyophilized, and 0.1 g dry weight was solubilized in lysis buffer containing 1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 1% PMSF, and 10 μg/ml leupeptin. Lysis was accomplished by discrete homogenization with 20 strokes in a type “A” Wheaton homogenizer. Lysates were clarified by centrifuging at 10,000 × g for 10 min. Clarified lysates were incubated for 2 h with 5 μl of R1 EGF receptor antiserum (Amersham). Immune complexes were harvested by addition of Staphylococcus aureus (Cowman strain) for 30 min. The immune complexes were then washed twice with a buffer containing 0.1% Triton X-100 and 150 mM NaCl in 10 mM sodium phosphate, pH 7.4. Pellets were resuspended in 25 μl of a solution containing 0.1% Triton X-100, 20 mM HEPES, and 100 μM sodium vanadate. The kinase reaction was initiated by addition of an equal volume of a solution containing 5 μCi of [γ-32P]ATP, 6 mM MnCl2, 0.1% Triton X-100, and 20 mM HEPES, pH 7.0. The reaction was allowed to proceed for 10 min at 25°C and terminated by addition of a buffer containing 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 150 mM NaCl, 5 mM EDTA, 1% trysol, 5 mM PMSF, 10 μg/ml leupeptin, 1 mM sodium vanadate, 5 mM sodium pyrophosphate, and 20 mM sodium phosphate, pH 7.4. Complexes were washed twice in the above buffer, and the phosphorylated proteins were resolved by SDS-polyacrylamide gel electrophoresis (8% acrylamide resolving gels) by the procedure of Laemmli (13).

Probes Used. Cloning of the EGF-r complementary DNA, HER-A64, has been described (14). EcoRI subclones were prepared and used as probes (Fig. 1). Preparation of the c-myc DNA probe, used as an internal control to compare relative amounts of DNA on the filters, has been described (15).

RESULTS

The EGF-r gene was amplified 64-, 8-, and 64-fold, respectively, in tumors obtained from Cases 1, 2, and 3 (Figs. 1 and 2). The extent of gene amplification in the tumors from Cases 1 and 3 was comparable to that seen in the DNA from the A431 cell line which is reported to be amplified 10- to 50-fold. Adjacent nontumorous tissues from Cases 1 and 3 were available and processed. The EGF-r gene was not amplified in these adjacent normal tissues. The signal intensity noted in these
AMPLIFIED EGF-r GENE IN PRIMARY BREAST CARCINOMA

Table I Characteristics of 21 patients with breast carcinoma analyzed for EGF-r gene structure abnormalities

<table>
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<tr>
<th>Case</th>
<th>Age</th>
<th>Menopausal status</th>
<th>Clinical stage</th>
<th>Nuclear grade</th>
<th>Histology</th>
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<th>PgR (fmol/mg)</th>
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<td>ILC</td>
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</table>
| *Inf. DC, infiltrating ductal carcinoma; Int. DC, intraductal carcinoma; ILC, infiltrating lobular carcinoma; ND, not done.

adjacent normal tissues was equal to that observed in the other tumor tissues analyzed (data not shown). The EGF-r gene was not rearranged in the DNA from Cases 1 and 3 when we used the restriction endonucleases EcoR1 and PstI and both EGF-r probes. There was not enough DNA from Case 2 to perform similar Southern blots.

To determine whether or not EGF-r gene amplification resulted in overexpression of the receptor protein product, an immunohistochemical analysis using a monoclonal antibody against the EGF-r was performed on the tumor tissue from Case 3 (Fig. 3). This analysis showed that more than 90% of the tumor nests showed intense positively stained cytoplasmic membranes. There were a few areas of tumor which were negative, which demonstrates the intratumoral heterogeneity of this breast tumor. There were no remaining available tissues from Cases 1 and 2 for this analysis. An additional 2 tumor tissues (Cases 7 and 18) without EGF-r gene amplification and their adjacent normal tissues were analyzed, and they did not react with the antibodies in this assay (data not shown).

To determine if the EGF-r proteins possessed functional tyrosine kinase activity, an immune complex kinase assay was performed on material from Case 3 as well as on 3 additional samples (Cases 7, 11, and 18) without EGF-r gene amplification. The results showed that, in tumor tissues, the normal M, 170,000 protein was phosphorylated. The level of kinase activity in Case 3 was similar to that described in the A431 cell line, a line known to have amplified EGF-r (Fig. 4, compare Lane 1 with Lane 7).

Cases 1 and 2 did not have axillary lymph node involvement, were postmenopausal, and had been taking conjugated estrogenic hormones for 9 mo and 6 yr, respectively, before their mastectomies. The tumors from Cases 1 and 2 were negative for ER and PgR, whereas the tumor from Case 3 was mildly positive for ER and PgR. In addition, the tumors from Cases 1 and 3 had Black's nuclear Grade 1 (16), which is consistent with a high degree of proliferation (Table 1).

DISCUSSION

EGF is a single chain polypeptide of 53 amino acids that is involved in the growth and differentiation of many types of...
AMPLICATED EGFR GENE IN PRIMARY BREAST CARCINOMA

3. Co

Fig. 2. Left, estimation of EGFR gene copy number by DNA dot blot analysis in the A431 squamous carcinoma cell line DNA (Lane 6), in the DNA obtained from the tumors of Cases 1 (Lane 1), 2 (Lane 3), and 3 (Lane 5), and in the DNA obtained from the normal breast tissues of Cases 1 (Lane 2) and 3 (Lane 4). The nylon filter was then boiled for 10 min and rehybridized with c-myb to confirm that equal amounts of DNA had been loaded onto each lane (right).

cells including mammary epithelium through a specific binding to a cell surface receptor (17, 18). EGFR has been detected in the breast cancer cell lines, as well as fresh human breast tumors, by a binding assay and immunohistochemistry with a monoclonal antibody (6-9). Although one-third to one-half of breast tumors are positive by these assays (19, 20), a structural analysis of EGFR gene in fresh human breast tumors thus far has not demonstrated that EGFR gene amplification is actually associated with high expression and high EGFR kinase activity. Our results indicate that one of the underlying molecular mechanisms of high expression of EGFR in breast cancer is gene amplification without gene rearrangement in a subset of human breast tumors. The EGFR gene was not rearranged, and the protein size was normal in our study, which is dissimilar to the findings in the A-431 squamous cancer cell line which showed amplification and rearrangement of the EGFR gene (14, 21).

Oncogene amplification in certain fresh tumor types is associated with unique clinical and pathological subsets. For instance, N-myc amplification in neuroblastoma was found in tumors resected from patients with advanced stages of disease and was associated with poor prognosis (22, 23). Similarly, EGFR gene amplification was reported in 4 of 10 patients with primary glial tumors of the brain (24). All 4 of these tumors were glioblastoma multiforme, representing clinicopathologically a more aggressive form of disease than astrocytomas that did not show gene amplification (24). Amplification of c-erbB2/neu gene in breast cancer was recently reported as a significant predictor of both overall and relapse-free survival in patients with breast cancer (25). In another report the presence of any altered protooncogene correlated with the clinical stage, tumor progression, and recurrence of breast cancer (26).

Fig. 3. Immunoperoxidase staining of frozen breast cancer tissue sections from Case 3 with the R1 anti-EGFR antibody. The arrows show tumor cell clusters which have antibody-positive staining of cytoplasmic membranes. x 100.

Fig. 4. Immune complex kinase assay of tumor tissue from Case 3 (Lane 7), of A431 cells (Lane 1), of normal tissue from Case 3 (Lane 6), of additional tumor tissues without EGFR gene amplification (Lanes 3, 5, and 9), and of the adjacent normal tissues from these same cases (Lanes 2, 4, and 8). Molecular weight markers are given on the right.
More recently, Sainsbury et al. (19) reported that the relapse-free and overall survival for patients with estrogen receptor-negative but EGF-r-positive breast cancer was significantly worse than for patients with both receptor-negative tumors. It is possible that EGF-r gene amplification and/or high EGF-r levels, as determined in this study, may be a biological predictor for poor prognosis in breast cancer. It is also important to point out that the tumors analyzed in this study were obtained from patients who had received no prior radiation therapy or chemotherapy. Hence, the amplification could not have been induced by either of these forms of anticancer therapy.

It is widely held that one of the characteristics of human breast cancer is its heterogeneity. The immunohistochemistry done in our study demonstrated this intratumoral heterogeneity and suggests that clones of cells within a tumor may harbor an amplified and overexpressed EGF-r gene, which may not be detected by methods which do not analyze individual cells.

Finally, just as elevated ER/PgR is predictive of hormonally sensitive breast cancer (27, 28), perhaps EGF-r gene amplification and/or high EGF-r levels indicate EGF-sensitive breast cancer. It is known that the addition of EGF in vitro inhibits the growth of A431 cells and the MDA-MB-468 cells, two cell lines known to have an amplified and overexpressed EGF-r (4, 21). EGF may, therefore, have a therapeutic role in patients with breast cancer who have EGF-r gene amplification and/or high EGF-r levels.

ACKNOWLEDGMENTS

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