Amphiregulin, Epidermal Growth Factor Receptor, and Estrogen Receptor Expression in Human Primary Breast Cancer

Susan LeJeune, Russell Leek, Elizabeth Horak, Gregory Plowman, Michael Greenall, and Adrian L. Harris

ABSTRACT

Amphiregulin is a recently described member of the epidermal growth factor family. Primary breast cancers were assessed for expression of amphiregulin by immunohistochemistry (111 cases), Northern, and/or dot blots (68 cases). Epidermal growth factor and estrogen receptors were measured in all cases. p53 and erbB-2 expression was assessed by immunohistochemistry for most cases. There was no association of these factors with amphiregulin expression, which was detected by immunohistochemistry in 40 of 111 cases. A significant association of amphiregulin expression assessed by Northern dot blots versus immunohistochemical staining was seen (P = 0.0016). Expression was not detected in adjacent nontumor tissue by immunohistochemistry. Amphiregulin was expressed in tumor epithelium, but not stromal or inflammatory cells. Expression was more common in lymph node positive cases (33 of 49; 47%) than lymph node negative cases (11 of 42; 26%; P = 0.04). The coexpression of epidermal growth factor receptor and amphiregulin in 35% of epidermal growth factor receptor positive cases raises the possibility of an autocrine loop in this subset of patients. Amphiregulin stimulates fibroblast growth and is up-regulated in breast cancer. A possible effect on tumor stroma may relate to the association with metastases.

INTRODUCTION

Peptide growth factors and their receptors are important in the regulation of the growth of human breast cancer (1). Estrogen regulated growth is partly mediated via stimulatory and inhibitory effects of these growth factors (2). Among the growth factor receptors relevant to human breast cancer are the EGF receptor (3, 4) and c-erbB-2 (5) which are both members of the EGFr family. The expression of these receptors has been related to prognosis in both lymph node positive (6-8) and negative cases (9, 10), and responsiveness to hormone therapy has been related to expression (11). ER and EGFr are inversely related in most studies (reviewed in Ref. 12).

Recently a ligand for erbB-2 was purified (13), and TGFα and EGFr are known ligands for EGF receptors (14). Both receptors are expressed in human breast tumors as are some of the ligands described above (15, 16). TGFα is often coexpressed with EGF receptors, providing the possibility of an autocrine loop (17).

However, there is an increasing family of EGFr-like receptors such as erbB-3 (18, 19) and also growth factors with homology to TGFα (20, 21). Recently, amphiregulin was cloned from a human breast cancer cell line (22). This protein was secreted in response to TPA and stimulated human fibroblasts (23). It inhibits some tumor cell lines (22). This protein was secreted in response to TPA and stimulated human fibroblasts (23). It inhibits some tumor cell lines (22). It could therefore be important in regulating breast cancer cell growth or in regulating stroma-epithelial interactions. This report therefore undertakes a more detailed analysis of amphiregulin association with human breast carcinoma. We have assessed amphiregulin expression by immunohistochemistry and by measuring mRNA levels in primary human breast cancer to investigate coexpression with EGFr and how it is related to ER expression (it was cloned from an ER positive breast cancer cell line, MCF-7).

We have demonstrated that amphiregulin is expressed in 36% of breast cancers but not associated with either EGFr or ER. It was not detected in adjacent nontumor breast tissue compared with the primary tumor. There was a significant association with regional lymph node metastases. Thus, expression is enhanced in breast cancer, although it did not correlate with other known prognostic factors in the primary tumor.

MATERIALS AND METHODS

Breast Tumor Samples

Tumors from patients with primary breast cancer were collected on ice in the operating room. Within 30 min, representative segments that appeared to be tumor macroscopically and excluding necrotic areas were excised, transferred to airtight cryovials, and snap-frozen in liquid nitrogen. Samples were stored in liquid nitrogen until required. RNA, DNA, cytosol, and membranes were prepared from each tumor. Prior to these preparations the tumors were minced finely with a scalpel and pulverized in a porcelain mortar chilled with liquid nitrogen.

RNA Extraction

Total RNA was isolated from the pulverized tumor samples using the acid guanidium-thiocyanate-phenol-chloroform method described by Chomczynski and Sacchi (24). Integrity of the RNA was analyzed on 1% agarose gels. Only RNA preparations with intact ribosomal bands were selected for subsequent analysis.

Control RNA was prepared from MCF-7 cells, cultured in Dulbecco’s modified essential medium supplemented with 10% fetal calf serum, and incubated for 16 h with 100 ng/ml TPA. These conditions have been shown to induce amphiregulin mRNA expression to a level of 18-fold higher than that of untreated MCF-7 cells (25).

Northern Blot Analysis

Twenty μg samples of total RNA were denatured in 1 M glyoxal, 50% v/v dimethylsulfoxide, and 10 mM sodium phosphate (pH 6.5) at 50°C for 45 min. RNA was fractionated on 1.2% agarose sodium phosphate gels and transferred to HyBond N⁺ nylon membranes (Amersham International) by capillary action using 0.025 M sodium phosphate (pH 6.8). RNA was UV-cross-linked to the nylon membranes using a Stratalinker (120 μ J). Glyoxal adducts were removed using boiling water. Transfer and integrity of RNA were checked by methylene blue staining.

Dot Blot Analysis

Dot blots were prepared as described by Potter and LeJeune (26). Briefly, 20 μg sample of total RNA were denatured with glyoxal and dimethylsulfoxide and deposited previously. RNA was transferred to nylon membranes (Pharmacia-Wallac) using a Schleicher and Schuell dot blot manifold with a format which allowed the membranes to be counted in the 120S β-plate flat bed scintillation counter (Pharmacia-Wallac) after hybridization, as described by Potter and LeJeune (26). RNA was UV cross-linked and deoxyxoylated as for Northern
blots. Tumors showing amphiregulin expression greater than 25% of that in TPA treated MCF-7 cells were considered to be amphiregulin positive. Results were normalized to actin for this calculation.

**Northern Hybridization Conditions**

Filters were prehybridized for 16 to 24 h at 60°C (AR and β-actin) or 50°C (glyceraldehyde-3-phosphate dehydrogenase) in 1.5 X SSPE, 1% SDS, 0.5 mg/ml denatured salmon sperm DNA, 10 X Denhardt’s solution, and 50% formamide. Hybridization was carried out under the same conditions except that 10% dextran sulfate was included in the hybridization buffer. CDNA probes were radiolabeled with α[^32p]dCTP by random priming to a specific activity of 3 X 10^6 dpm/μg according to the method of Feinberg and Vogelstein (27). AR cDNA probe was a EcoRI fragment of 1.1 kilobase containing the complete AR cDNA sequence except for 100 base pairs from the 3’ untranslated region (25). Human β-actin probe was obtained from Clontech Laboratories, Inc., and contained the complete 2-kilobase human β-actin cDNA.

After hybridization filters were washed in decreasing concentrations of standard saline citrate (2 X; 0.5 X; 0.1 X) containing 0.1% SDS followed by a final stringent wash at 60°C in 0.1 X standard saline citrate containing 1% SDS. Northern blots were autoradiographed using Kodak X-AR film at -70°C with intensifying screens. Dot blots were counted in the flat bed liquid scintillation counter (Pharmacia-Wallac) as described by Potter and LeJeune (26).

**DNA Extraction**

Pulverized tumor samples were incubated overnight at 37°C in 50 mM Tris-HCl (pH 8), 10 mM EDTA, 0.5% sarkosyl, and 100 μg/ml proteinase K. Ribonuclease A was added to 20 μg/ml and incubation continued for an additional 30 min. The DNA was extracted with one volume each of phenol/phenol/phenol. The homogenate was centrifuged at 3000 rpm for 10 min. The supernatant was resuspended in buffer and stored at -80°C until assayed for EOF; the supernatant (cytosol) was made 0.002 M with respect to dithiothreitol.

**Southern Hybridization Conditions**

Prehybridization and hybridization was for 16 to 24 h at 72°C in 10% dextran sulfate, 10 X Denhardt’s solutions, 1.5 X SSPE, 2% SDS, and 400 μg/ml denatured salmon sperm DNA. CDNA probe preparation washing and autoradiography were as for Northern filters except that the final stringent wash was carried out at 55°C. The β-globin probe was a 0.9-kilobase EcoRI-BamHI cDNA fragment obtained from B. Morley.

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**Image Analysis**

Autoradiograms of Southern blots were scanned using a Bio-Image image analyser (Milligen Biosearch). To correct for differences in DNA loading and transfer efficiency, filters were hybridized to a β-globin probe and the ratio of amphiregulin/β-globin was calculated. Placental DNA was assumed to have a single copy of amphiregulin. The tumors studied had a mean ratio of 1.3 ± 0.4 (SD) times that of the ratio amphiregulin/β-globin in placenta. Amplification was considered to be a ratio of 2 times that of placenta.

**Receptor Assays**

**Tissue Preparation.** All procedures were carried out at 0–4°C. Pulverized tumors were further homogenized in a ratio of 1:20 (w/v) in buffer (0.02 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-0.00075 M EDTA-0.001 M benzamidine-0.0005 M phenylmethylsulfonyl fluoride-1 μg/ml ovomucoid, pH 7.4; 20°C) using 2 10-second bursts of a Polytron homogenizer at setting 6. The homogenate was centrifuged at 3000 rpm for 10 min. The supernatant was centrifuged at 37,000 rpm for 40 min. The pellet (crude membrane fraction) was resuspended in buffer and stored at -80°C until assayed for EGF; the supernatant (cytosol) was made 0.002 M with respect to dithiothreitol.

**Amphiregulin**

Fig. 1. Amphi-regulin expression in breast tumors. Northern blot of total RNA isolated from the following sources: Lanes 1, MCF-7 cells; Lanes 2, MCF-7 cells treated with 100 ng/ml TPA for 16 h; Lanes 3–5, 7, and 8–18, inclusive primary breast tumors; Lanes 5–7, other breast disease (benign cystic disease). Reprobing was performed with actin and the Northern blot is shown superimposed below the appropriate cases.

**β-actin**

Fig. 2. Correlation of quantitative Northern blots with β-plate counts for amphiregulin. Tumor RNA was assayed by the β-plate counter method or run on gels and Northern blotted. The latter were quantitated by image analysis densitometry. Results were expressed in each case as the percentage of level induced in MCF-7 cells.

**Estrogen Receptor Assay.** The ER content of the tumors was determined using the dextran-coated method (31). Tumor specimens were considered ER positive if they contained at least 10 fmol of specific binding sites/mg of cytosolic protein.

**EGF Receptor Assay.** EGF was iodinated by the iodogen method (32). The EGFRe receptor activity was determined by scintillation counter (Phannacia-Wallac) as described by Potter and LeJeune (26).
membranes (50–100 µg protein) were incubated for 60 min at 25°C with 125I-EGF (final concentration, 1 nM) in the presence and absence of unlabelled EGF (final concentration 100 nM) to correct for nonspecific binding. Membranes were pelleted by centrifuging at 14,000 rpm for 7 min at 4°C, washed with ice-cold phosphate-buffered saline containing 0.2% bovine serum albumin, recentrifuged as above, and counted in a Beckman gamma 5500B spectrometer. A cutoff value of 20 fmol/mg protein was used to differentiate between receptor positive and negative tumors.

Immunohistochemistry. The criteria for positivity have previously been reported (35). The p53 antibody detects an epitope in mutated p53 and also wild-type when overexpressed in a conformation associated with proliferation.

RESULTS

Northern Blots. 13 primary tumors were studied by Northern blotting. A 1.4-kilobase transcript was detectable (Fig. 1). A comparison of the Northern blots and quantitative dot blots assayed by scintillation counting on the β-plate counter showed a significant correlation (P < 0.0001; r = 0.87) and no significant difference in ranking (Fig. 2). In each case, results were quantified as % of level induced in MCF-7 cells by TPA.

Quantitative RNA Dot Blots. Sixty-eight tumors were assessed by a quantitative method of direct counting on filters using a flat-bed scintillation counter. Twenty-two of 68 tumors expressed levels 25% of the levels induced in MCF-7 cells by TPA, or greater. There was no association of amphiregulin mRNA expression with ER, EGFr, or combinations of these receptors (Fig. 3; Table 1).

Southern Blots. Eight high expressors of RNA for amphiregulin (>25% of TPA induced level in MCF-7) and 8 tumors with levels 2-fold or less above background were analyzed to see if high expression was due to gene amplification. No amplification was detected (Fig. 4). However, a polymorphism for EcoRl was found, one pattern consisting of bands of 7.9, 5.9, and 3.25 kilobases. The other comprised bands of 5.9, 4.8, 3.6 and 3.25 kilobases. The polymorphism is within the 7.9-kilobase band giving rise to bands of approximately 4.8 and 3.6 kilobases. Amongst the 16 tumors, 10 were homozygous for the 7.9-kilobase band, 1 for the 4.8- and 3.6-kilobase bands, and 4 were heterozygous and 1 had degraded DNA. There are also 4 cases of benign disease shown and a placent control. One of the benign cases is heterozygous (case 17). The placenta is homozygous for the 7.9-kilobase band, 1 for the 4.8- and 3.6-kilobase bands, and 4

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<th>Table 1 Amphiregulin mRNA expression and immunohistochemistry staining compared with EGFr and ER expression</th>
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<td>ER was measured by ligand binding and 5 fmol/mg protein or greater was considered positive. For EGFr 20 fmol/mg or greater by ligand assay was positive</td>
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enzyme linked immunosorbert assay and immunostaining of paraformaldehyde fixed cells, this monoclonal antibody has been shown to be specific to human AR and does not cross-react to EGF, TGFα, HB-EGF, or to rodent AR. By enzyme linked immunosorbert assay, monoclonal antibody 6RIC2.8 shows linearity from 20-2000 pg/ml but does not work well in serum containing samples. In frozen tissue sections this epitope is stable to paraformaldehyde, acetone, and gluteraldehyde, although when using other conditions it is labile.

Positive and negative controls initially consisted of TPA induced and control MCF-7 cells. Once positive tumors were identified, these were subsequently used as controls. Negative controls consisted of sections in which the first antibody was omitted. Staining ranged from 25 to 100% of tumor cells. Differing intensity or proportions of cells were all included as positive.

erbB-2 and p53 expression were assessed using the antibodies TAI and Ab240, respectively, on frozen sections. The antibody CB11 was used on paraffin sections to stain erbB-2. The criteria for positivity have previously been reported (35). The p53 antibody detects an epitope in mutated p53 and also wild-type when overexpressed in a conformation associated with proliferation.

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Fig. 3. Dot blots of breast cancer RNA, classified by ER and EGFr status. Labels to the right of each dot indicate sample number. A. R positive tumor RNA probed with amphiregulin. Samples 1-16 represent EGFr negative tumors. Samples 17-27 represent EGFr positive tumors. B. ER positive tumor RNA probed with β-actin. C. ER negative tumor RNA probed with amphiregulin. Samples 28-38 represent EGFr negative tumors. Samples 39-52 represent EGFr positive tumors. D. ER negative tumor RNA probed with β-actin.
Fig. 4. Southern blots of amphiregulin in high and low expressing tumors. Southern blot of tumor DNA digested with EcoRI. Lanes 1-8, tumors showing high expression; Lanes 9-16, tumors showing low expression; Lanes 17-20, benign breast disease; Lane 21, placenta. Lanes 2, 3, 5, and 10 are heterozygous tumors; other lanes up to 16 are homozygous tumors.

![Amphiregulin Southern Blot](image)

![β-globin Southern Blot](image)

Fig. 5. Immunohistochemical staining of breast cancer for amphiregulin. A, tumor staining present but none in the stroma (×10). B, higher power view (×25). C, negative tumor.

Fig. 6. Amphiregulin staining of tumor and adjacent normal breast. The normal ducts do not stain but tumor does.

of cells. There was no direct correlation of staining intensity with degree of staining.

In 32 cases there was adjacent nontumorous breast and in no cases did this stain with the antibody (Fig. 6). In 5 of these cases the tumors were positive. Thus, there was a significantly higher frequency of expression of amphiregulin in tumor versus adjacent breast tissue ($P < 0.01$; $\chi^2$ test with Yates correction). The frequency of expression of AR in tumors near normal epithelium was lower than in samples without adjacent tumor. This may reflect sampling or have implications for AR function. Immunocytochemistry was concordant with Northern dot blots in 80% of cases. In 11%, cases were positive by dot blots only, and in 9% immunohistochemistry only.

**AR-positive Tumors, ER, and EGFr Expression.** Expression of ER and EGFr was assessed in the 111 cases where AR staining was also available. AR expression was in a similar proportion of ER+ cases, EGFr+ cases, or combinations of ER and EGFr (Table 1). There was an inverse relationship of ER and EGFr 17 of 52 (32%) of ER+ were EGFr+, compared with 29 of 59 (49%) of ER cases.

**Association with Other Pathological Variables and the Oncogenes p53 and erbB-2.** The frequency of amphiregulin staining was similar amongst the major histological subtypes (29 of 78 ductal, 37%; 4 of 16 lobular, 25%; 7 of 17 others, 41%). Although not available on all cases, p53 and erbB-2 expression assessed by immunocytochemistry showed no correlation with AR expression (Table 2). Similarly, sizes 2 cm were not associated with AR expression.

**DISCUSSION**

The AR gene was described in 1990 (25) and in a preliminary assessment of normal human tissues that express the transcript Plow-
man et al. (25) showed that the highest levels were in ovary and placenta, with lower levels in colon, breast, lung, pancreas, spleen, testis, and cardiac muscle. There has been no previous description of amphiregulin expression in breast cancers or a number of normal breast specimens.

Our study shows that amphiregulin is expressed immunohistochemically more frequently in breast cancer than in normal breast tissues, 37 versus 0% of cases. It is commonly expressed in tumors but not the adjacent nontumorous breast. This suggests that AR transcription and expression is up-regulated in transformed cells. In 6 of 11 human breast cancer or breast cell lines, AR transcripts were detected (25). Although the highest levels were in ER positive cell lines, transcripts were also present in ER negative breast cell lines. There was no association of transcripts or protein expression with ER status in primary breast cancers.

Amphiregulin was detected in another study in mammary breast cells (strain 161) and much lower levels in 2 breast cancer cell lines (34). Thus, there is a wide variation expressed in cell lines.

In a range of human cell lines, including 5 breast cancer cell lines, TPA up-regulated AR mRNA up to 18-fold, and some cell lines showed high constitutive expression (25). In some of the primary tumors in our study expression of RNA was even higher than that induced in cell lines. It was shown in these highest expressing tumors that AR staining was also present. Whether this upregulation in vivo is via a protein kinase C pathway is not possible to elucidate, but it was not due to gene amplification. Perhaps a gene that has such a wide range of inducibility can be overexpressed if it has a role in cancer by changes in transcription regulation pathways which are already available. Recently growth factors that act via the protein kinase C pathway, such as bombesin (36) and vasopressin (37), have been shown to affect breast cancer cell line growth.

Those tumors with the highest RNA expression for AR also stained for AR protein. However, in some cases with detectable levels of transcripts, staining could not be detected. Since there were positive immunohistochemical controls and adjacent sections reacted with other antibodies for membrane or nuclear proteins, although a labile epitope could explain some losses, it is unlikely that this was due to degradation of AR protein. Similarly, cases with low AR transcripts did not have excessive RNA degradation assessed by staining gels with ethidium bromide and controls blots with actin. It is likely that regulation of protein expression occurs at a transcriptional and posttranscriptional level, as has been shown in MCF-7 cells (25).

Other possibilities are that there is a variable normal tissue or stromal component diluting RNA giving some cases positive by immunohistochemistry but not by RNA analysis. Aberrant forms of amphiregulin may exist but not be detectable by the antibody used. However, 6R1C is highly specific for all known forms of AR (secreted high molecular weight variants, transmembrane and cell associated). Each method of assessment has its own sensitivity, so some tumors with low levels of RNA classified as low AR may still have enough to produce AR protein.

Amphiregulin can inhibit the growth of some breast cancer cells. If this also occurred in vivo it is not clear why high expression should occur in carcinomas. However, other growth factors that are inhibitory to normal mammary epithelial cell lines and breast cancer in vitro are commonly expressed in primary cancers, e.g., TGFβ (38-40). A potential role is in stimulation of the production of matrix, stromal cell proliferation, or angiogenesis. Similarly stromal cells may influence the growth of the mammary epithelial cells (41, 42). EGF modulates extracellular matrix composition produced by breast stromal cells (42). Since amphiregulin stimulates fibroblast growth in vitro, the expression of proteolytic enzymes in the stromal cells and the relation to amphiregulin expression would be of interest to assess. A novel protease, stromelysin-3, is expressed in stromal cells of primary human breast cancers and amphiregulin is a candidate growth factor for regulation of this protease (43).

Amphiregulin is a ligand for the EGF receptor and cross-talk between the EGFR and erbB-2 has been shown, with increase in transforming potential (12, 44). Thirty-five % of EGFr positive tumors showed amphiregulin staining, so a potential autocrine loop exists in this subgroup of patients. Staining was often on the membrane, so a juxtacrine mechanism is also possible (45). Whether this is a particularly poor prognosis group will be assessed in future follow-up.

Ciardiello et al. (46) showed high expression of amphiregulin in tumors 15 of 30 primary colon tumors but only 4 of 27 normal adjacent tissues. Stromal cells were negative. These results are similar to those in the breast cancers. In a more recent publication (47), it was shown that normal mucosa from patients without colon cancer did express amphiregulin, so it may be down-regulated in dysplastic tissue. However, the normal breast epithelium did not appear dysplastic. Although amphiregulin expression alone did not associate with any known prognostic marker in the primary breast tumors, its high expression in a subgroup of cancers suggests a biological role. The association with node metastasis needs further studies. In the EGFr positive cases AR may interact adversely but follow-up is too early to assess. Further elucidation of the role this protein may allow novel approaches to therapy, e.g., by modulating tumor-stroma interactions or heparin-like drugs (35).

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