Epidermal Growth Factor Receptor-negative Tumors Are Predominantly Confined to the Subgroup of Estradiol Receptor-positive Human Primary Breast Cancers

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ABSTRACT

A total of 725 human primary breast tumor biopsy samples were analyzed for epidermal growth factor receptor (EGFR) content, using a multiple-point EGFR assay standardized in accordance with the recommendations of the European Organization for Research and Treatment of Cancer Receptor Study Group.

After the establishment of a lower cell membrane protein threshold of 0.2 mg of membrane protein per ml of assay buffer, the results of 27% (194 samples) of the EGFR determinations were excluded from the study because of insufficient assay membrane protein content. Of the remaining 531 breast tumor biopsy samples, 57% (302 samples) were shown to be EGFR positive by Scatchard analysis, with a median value of 40 fmol/mg of membrane protein.

Of the breast tumor biopsy samples, 72% (380 samples) were estrogen receptor (ER) positive, and 65% (344 samples) were progesterone receptor (PGR) positive. EGFR positivity was found in 46% (173 of 380) of ER-positive and in 85% (129 of 151) of ER-negative breast tumor biopsy samples (P < 0.0001), as well as in 49% (168 of 344) of PGR-positive and in 72% (134 of 186) of PGR-negative breast tumor biopsy samples (P < 0.0001). Mean EGFR levels in ER-positive breast tumor biopsy samples were lower than those in ER-negative ones, 40 ± 31 (SD) against 72 ± 55 fmol/mg of membrane protein (P < 0.0001). Similarly, mean EGFR levels in PGR-positive breast tumor biopsy samples were lower than those in PGR-negative ones, 41 ± 29 against 70 ± 56 fmol/mg of membrane protein (P < 0.0001). Both EGFR positivity and EGFR levels decreased with increasing steroid hormone receptor levels. A multivariate analysis showed only ER to be independently associated with EGFR.

INTRODUCTION

In 1962 Cohen (1) reported the isolation from the mouse submaxillary gland of a protein accelerating incisor eruption and eyelid opening in the newborn animal. The protein was shown to induce epidermal basal cell proliferation and accordingly called EGF (2, 3).

The biological effects of EGF (and EGF-related proteins) are mediated through a specific cell membrane-bound receptor (4). The receptor, a Mr 170,000 transmembrane protein, comprises an extracellular ligand-binding domain which is linked by a short hydrophobic transmembrane stretch to a cytoplasmic domain expressing an intrinsic tyrosine kinase activity believed to be responsible for post-receptor signaling (5-7).

In 1987 Sainsbury et al. (8) reported the presence of EGFRs to be indicative of poor prognosis in human primary breast cancer. Since then various authors, using different techniques in preparing tumor cell membrane fractions and different assays for EGFR, have reported differently on the significance of EGFR as a prognostic factor. This study focuses on the estimation of EGFR using a ligand binding assay standardized in accordance with the 1991 recommendations of the EORTC Receptor Study Group and on the associations between EGFR and steroid hormone receptors in breast tumor biopsy samples (Footnote 3; Ref. 9).

MATERIALS AND METHODS

Stored cell membrane preparations of 341 human primary breast tumor samples obtained between October 1987 and August 1988 and 384 cell membrane preparations of tumor biopsy samples obtained between May 1989 and May 1990 were analyzed for EGFR content from January 1990 and onward. All cell membrane preparations were obtained during routine processing of tumor biopsy samples for steroid hormone receptor assays.

Steroid Hormone Receptor Assays. These were performed in (both series) within 4 wk following surgery, utilizing the dextran-coated charcoal method and multiple-point technique as recommended by the EORTC (10). In short, after pulverization in the frozen state the tumor biopsy sample was homogenized by means of a microdismembrator and dissolved in 1 ml of assay buffer (0.01 M phosphate buffer, pH 7.4, containing 0.01 M K2HPO4/KH2PO4, 0.0015 M dipotassium EDTA, 0.003 M NaN3, 0.01 M monothioglycerol, and 10%, v/v, glycerol). The homogenate was centrifuged for 15 min at 800 x g, 4°C, to spin down nuclei and other coarse cell fragments. The supernatant was centrifuged for 60 min at 105,000 x g, 4°C, and subsequently used for ER and PgR determinations. Cut-off values for ER and PgR were set at 10 fmol/mg of cytosolic protein. The cell membrane pellets were rinsed with 1 ml of EGFR assay buffer (0.02 M phosphate buffer, pH 7.4, containing 0.15 M NaCl and 70 μg/ml of Bacintracin) and stored at −80°C under an aliquot of assay buffer during periods of time varying from 1 wk to 3 yr.

Epidermal Growth Factor Receptor Assays. These were in essence similar to those described previously (9). In short, the stored cell membrane pellets were resuspended in 1.1 ml of assay buffer by means of ultrasound bursts (MSE Soniprep-150; nominal frequency, 23 KHz; amplitude, 10 μm) for 10 s, on ice. A 100-μl aliquot was taken for membrane protein determination (Coomassie brilliant blue method in preparing tumor cell membrane fractions and different assays for EGFR, have reported differently on the significance of EGFR as a prognostic factor. This study focuses on the estimation of EGFR using a ligand binding assay standardized in accordance with the 1991 recommendations of the EORTC Receptor Study Group and on the associations between EGFR and steroid hormone receptors in breast tumor biopsy samples (Footnote 3; Ref. 9).

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incubated for another hour at 20°C, during which they were shaken 4 times. Subsequently the tubes were centrifuged for 2 min at 800 x g, 20°C. The supernatant (containing the unbound ligand) was decanted. The HAP pellet was washed 2 times with assay buffer (20°C) before radioactivity was assayed in a γ-ray counter. A human placental membrane preparation containing a fixed amount of EGFR served as a positive control for 125I-mEGF—EGFR binding. Receptor values were calculated by Scatchard analysis and expressed as fmol/mg of membrane protein (12). EGFR analyses resulting in interpretable Scatchard plots were regarded as positive. Plots were regarded as interpretable if the correlation coefficient obtained from the Scatchard plot, using a minimum of 6 of the 8 dose points, was found to be higher than 0.83 and if specific binding accounted for less than 50% of total binding.

Statistics. Nonparametric test statistics were applied, since the receptor data were not normally distributed. Parametric tests were performed only after logarithmic transformation of data. Associations between variables were assessed by the Spearman rank correlation test (the correlation coefficient denoted as Rs and the significance level as P<). Homogeneity between groups was tested nonparametrically by means of the Wilcoxon two-sample test (Kruskall-Wallis test for multiple groups) [x² denoted as x² k,kw, degrees of freedom as d.f., and level of significance as P k,kw]. For qualitative relations the x² test for contingency tables was used (x² denoted as x², degrees of freedom as d.f., and P as P x). For multivariate analysis of quantitative receptor data a multiple regression analysis was applied (level of significance denoted as P reg). The categorical modeling procedure served as a matrix for a multivariate analysis of the qualitative receptor data (level of significance denoted as P cat). Test results were regarded as significant at the P < 0.01 level. All calculations were performed using SAS (Statistical Analyzing System) statistical software (13).

RESULTS

The two series of breast tumor biopsy samples were found not to differ in ER, PgR, and EGFR status (Table 1). Subsequent analysis did not reveal any association between the varying length of time during which the cell membrane pellets were kept at −80°C and their assessed EGFR content (R<sub>s</sub> = −0.02, n = 725; P<sub>s</sub> = 0.51). Therefore the estimations relative to the retrospective and prospective series were combined for further analysis. Univariate analysis showed the EGFR levels to be positively associated with cell membrane protein levels (R<sub>s</sub> = 0.15, n = 725, P<sub>s</sub> = 0.0001), with declining percentages of EGFR positives at the lower cell membrane protein levels (x² = 64, d.f. = 10, n = 725, P<sub>x</sub> < 0.0001), indicating that at these levels EGFR concentrations submerged the lower limit of assay sensitivity, resulting in false negatives (see Fig. 1). When EGFR estimations associated with cell membrane protein levels up to 0.2 mg/ml were disregarded, the remaining estimations showed no further association between EGFR data and cell membrane protein levels (R<sub>s</sub> = −0.01, n = 531, P<sub>s</sub> = 0.74 and x² = 9.1, d.f. = 8, n = 531, P<sub>x</sub> = 0.34). Accordingly a membrane protein level of 0.2 mg/ml was adopted as a threshold. In consequence, 27% (194 samples) of the EGFR estimations were rejected for the purpose of the present study.

Analysis of the 531 remaining sets of data revealed that 57% (302 samples) of the tumor cell membrane preparations contained specific, saturable, high-affinity binding sites for EGF, total EGFR levels ranging from 3 to 3600 fmol/mg of membrane protein, with a median value of 40 fmol/mg of membrane protein (median K<sub>d</sub> = 0.5 nM; range, 0.04 to 2.6 nM). Again, no association was found between EGFR positivity or EGFR levels and duration of storage at −80°C, ranging from 1 wk to 3 yr (x² = 10.7, d.f. = 5, n = 531, P<sub>x</sub> = 0.06 and x² kw = 1.35, d.f. = 5, n = 302, P = 0.92; Fig. 2).

 Seventy-two % (380 of 531) of the tumor biopsy samples were ER positive, and 65% (344 of 530) were PgR positive. EGFR positivity was found in 46% (173 of 380) of ER-positive and in 85% (129 of 151) of ER-negative breast tumor biopsy samples (x² = 70.1, d.f. = 1, n = 531, P<sub>x</sub> < 0.0001) as well as in 49% (168 of 344) of PgR-positive and in 72% (134 of 186) of PgR-negative breast tumor biopsy samples (x² = 26.5, d.f. = 1, n = 530, P<sub>x</sub> < 0.0001). Mean EGFR levels in ER-positive breast tumor biopsy samples were significantly lower than they were in ER-negative ones, 40 ± 31 (mean ± SD) against 72 ± 55 fmol/mg of membrane protein (x²<sub>kw</sub> = 33.1, d.f. = 1, n = 302, P<sub>kw</sub> < 0.0001). Similarly, mean EGFR levels in PgR-positive breast tumor biopsy samples were significantly lower than they were in PgR-negative ones, 41 ± 29 and 70 ± 56 fmol/mg of membrane protein (x²<sub>kw</sub> = 21.4, d.f. = 1, n = 302, P<sub>kw</sub> < 0.0001).

In addition, the percentage of EGFR-positive cell membrane

Table 1  Distribution of steroid hormone and EGFR receptors over the retrospective and prospective series

<table>
<thead>
<tr>
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<th>ER+</th>
<th>PgR+</th>
<th>EGFR+</th>
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<tr>
<td></td>
<td>No. of patients on study</td>
<td>n</td>
<td>Median&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>Retrospective</td>
<td>341</td>
<td>254 (74)&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>Prospective</td>
<td>384</td>
<td>283 (74)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>109</td>
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<td></td>
<td>P</td>
<td>0.81&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.58&lt;sup&gt;c&lt;/sup&gt;</td>
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</tbody>
</table>

<sup>a</sup> Steroid hormone receptor values expressed in fmol/mg of cytosolic protein.
<sup>b</sup> EGFR values expressed in fmol/mg of membrane protein.
<sup>c</sup> Numbers in parentheses, percentage of number of patients on study.
<sup>d</sup> P<sub>x</sub>.
<sup>e</sup> P<sub>kw</sub>.

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Fig. 1. Relation between cell membrane protein (expressed as mg/ml, displayed as bar end points) and the percentages of EGFR positivity (bars representing percentage of numbers displayed in the bars).
preparations decreased with increasing ER levels ($\chi^2 = 50.8$, d.f. = 6, $n = 531$, $P < 0.0001$; Fig. 3A) as did the EGFR levels ($\chi^2_{kw} = 50.8$, d.f. = 6, $n = 302$, $P_{kw} = 0.0001$; Fig. 3B). Likewise, EGFR positivity and EGFR levels were shown to be negatively associated with PgR levels ($\chi^2 = 45.2$, d.f. = 6, $n = 530$, $P < 0.0001$ and $\chi^2_{kw} = 34.2$, d.f. = 6, $n = 302$, $P_{kw} = 0.0001$; Fig. 3, C and D).

Univariate analysis of the EGFR data in relation to the combined steroid hormone receptor status of the tumor biopsy samples showed that the absence or presence of ER was the factor associated with EGFR positivity ($\chi^2 = 70.1$, d.f. = 3, $P < 0.0001$; Fig. 4/Í).

EGFR levels differed significantly between the ER+/PgR+ and the ER−/PgR− subgroups ($\chi^2_{w} = 27.0$, d.f. = 1, $n = 247$, $P < 0.0001$) but did not differ between the ER+/PgR+ and ER+/PgR− subgroups ($\chi^2_{w} = 0.5$, d.f. = 1, $n = 173$, $P = 0.47$) nor between the ER−/PgR− and ER−/PgR+ subgroups ($\chi^2_{w} = 1.52$, d.f. = 1, $n = 129$, $P = 0.21$) nor between the ER+/PgR− and the ER−/PgR+ subgroups ($\chi^2_{w} = 1.44$, d.f. = 1, $n = 55$, $P = 0.23$; Fig. 4B).

Multivariate analysis introducing EGFR (status and levels) as the dependent variable and ER, PgR as independent variables showed that only ER was independently associated with EGFR ($P_{CAT}$, ER $< 0.0001$, PgR = 0.46; $P_{REG}$, ER $< 0.0001$, PgR = 0.72), implying that the association between PgR and EGFR is an exponent of the strong association between PgR and ER ($R_s = 0.63$, $n = 530$; $P < 0.0001$).

**DISCUSSION**

The data presented demonstrate that EGFR remains stable for at least 3 yr in cell membrane preparations stored at −80°C, a finding which legitimates the use of archived membrane preparations for the purpose of retrospective clinical studies on EGFR.

Our study shows that measurement of EGFR by means of a ligand binding assay is apt to give a false-negative outcome in cases where the cell membrane protein level falls below a certain threshold (in our study, using the multiple-point HAP assay, 0.2 mg/ml). The amount of cell membrane protein obtained after processing a breast tumor biopsy sample is related to its wet weight ($R_s = 0.48$, $n = 725$, $P < 0.0001$). The adoption of a cell membrane protein threshold of 0.2 mg/ml implies that tumor biopsy samples less than 300 mg wet weight do not qualify for EGFR determination by the multiple-point assay technique.

The percentage of EGFR positivity obtained in our study (57%) is the second highest hitherto reported (Table 2). This
pressed as fmol/mg of membrane protein ± numbers displayed in the bars) and mean positivity (A) (bars representing percentage of steroid hormone receptor status of human primary breast cancer and percentages of EGFR positivity (A) [bars representing percentage of numbers displayed in the bars] and mean EGFR concentrations of positives (B) (expressed as fmol/mg of membrane protein ± SEM).

![Graph A](image)

![Graph B](image)

**Table 2. A survey of the ligand binding EGFR literature**

<table>
<thead>
<tr>
<th>No. of patients on study</th>
<th>ER+</th>
<th>ER-</th>
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<tr>
<td></td>
<td>EGFR+</td>
<td>EGFR-</td>
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<tr>
<td>Bolufer et al. (16)</td>
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<td>Battaglia et al. (28)</td>
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<td>Sainsbury et al. (8)</td>
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<td>47</td>
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<tr>
<td>Perez et al. (19)</td>
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<tr>
<td>Toi et al. (20)</td>
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<td>Cappelletti et al. (22)</td>
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<td>This report</td>
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<tr>
<td>Fockens et al. (25)*</td>
<td>195</td>
<td>78</td>
</tr>
</tbody>
</table>

* Numbers in parentheses, percentage of patients on study.

* Cut-off level for EGFR set at 0.5 fmol/mg of membrane protein.

high percentage may be partly attributable to our exclusion of false negatives associated with cell membrane protein levels below 0.2 mg/ml. The median EGFR level in our study (40 fmol/mg of membrane protein) also exceeds most of the values reported. This relatively high median finds an explanation in the multiple-point assay technique applied, permitting the extrapolation of EGFR binding data to the abscissa of the Scatchard plot, a proceeding resulting in higher, more accurate, receptor levels than obtainable by the single-point technique, the technique most widely used. The high EGFR levels are probably also attributable to our using HAP instead of ultracentrifugation to separate receptor-bound and free ligand (9).

In this study we found 85% (129 of 151) of the ER-negative breast tumor biopsy samples to be EGFR positive. This finding is in agreement with in vitro studies in which ER-negative human breast cancer-derived cell lines were found to constitutively express EGFR (14, 15) but is significantly higher than the percentages reported in the current EGFR literature (Table 2). In the ER-positive subgroup we found that 46% (173 of 280) of the cell membrane preparations were EGFR positive. Furthermore, both EGFR positivity and EGFR levels were shown to be negatively associated with steroid hormone receptor levels. These data confirm earlier reported negative associations between ER and EGFR (8, 16-27) as well as between PgR and EGFR (22-28), associations only twice disputed in the literature (29, 30). Our data also show that the association between PgR and EGFR is not an independent one but is an exponent of the strong association between ER and PgR.

The percentages of EGFR positivity as reported in the literature vary from 22% to 67% (Table 2). Differences in assay methodology and tumor biopsy sample selection seem to offer plausible explanations for this variation. However, in the case of tumor biopsy sample selection, one would expect, given the established negative association between ER and EGFR, to find the higher percentages of EGFR positivity in studies reporting lower percentages of ER positives. Since this is not the case, methodological differences are the most likely explanation for the observed variation.

The authors consider the standardization of EGFR assays (as established by the EORTC) of the utmost importance to attain uniformity and comparability of EGFR data, a prerequisite when trying to determine the significance of EGFR as a prognostic marker in human breast cancer and its role in tumor biology.

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