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Estimation of the Epidermal Growth Factor Receptor by the Hydroxyapatite Method in Human Breast Cancer[1])

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Summary: Results of epidermal growth factor (EGF) receptor in human breast tumours show large variations, mainly due to the lack of standardization of the assays. Our EGF receptor values are higher than those reported previously which may be due to the use of the hydroxyapatite to separate bound and non-bound ligand in a radioligand assay. We found EGF receptors in 58% (103/178) of the tumours (EGF receptor levels: 3 to 625 fmol/mg of membrane protein, $\bar{x}$ = 33.3, median = 17.4), with a median K_d of 0.642 nmol/l. There was an inverse correlation between EGF receptors and estrogen receptors ($r = -0.215$, $p = 0.00002$, *Kendall* correlation). There was a difference between EGF receptor content in grade II ($\bar{x}$ = 16.9) and grade III tumours ($\bar{x}$ = 59.3) ($p = 0.027$), but not between histopathological types and lymph node status.

The relevance of EGF receptor largely depends on the reliability of its determination. The standardized EORTC methodology is a reproducible alternative which will expand EGF receptor determination and permit comparability of data between laboratories.

Introduction

Epidermal growth factor (EGF) is a 53 amino acid polypeptide (M_r = 6000) isolated from the mouse submaxillary gland (1). The physiological role of EGF, although not yet completely understood, seems to be to regulate both the proliferation and/or differentiation *in vitro* and *in vivo* of a wide variety of cell types (2). Particularly, it is known to stimulate proliferation of human breast cancer cells in culture (3, 4). The biological effects (paracrine or autocrine) of EGF are mediated through high affinity binding to the specific cell-membrane receptor (EGF receptor) (2). This receptor is a M_r = 170 000 membrane glycoprotein characterized by tyrosine kinase activity, and its intracellular domain is homologous to sequences of the oncogen c-erbB-2 (5, 6), and also serves as specific receptor for transforming growth factor-α (TGFα) (7).

The presence of EGF receptor in human breast tumours has been reported by many groups (8). The majority of these studies has shown that the presence of EGF receptor is inversely correlated with steroid receptor content and provides valuable, although controversial, prognostic information in breast cancer (9, 10). There is still no consensus on EGF receptors role in prognosis, but recent studies have supported the hypothesis that EGF receptor may be useful for predicting response to endocrine therapy (11) or as a target for new types of treatment (12). The most widely used method of receptor quantitation has been radioligand assay in which ^{125}I-labelled EGF is incubated with tumoural membranes. High affinity binding specific for EGF has been reported in 16% to 91%

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of primary breast tumours at levels ranging from 1 to 3600 fmol/mg of protein, with a median of the distribution ranging from 1.1 to 40 fmol/mg of protein in the various studies reviewed (8). These large variations can be explained by the absence of standardization of tissue-processing and assay conditions (13), and the heterogeneity of clinical material tested as well.

Our study focuses on the estimation of EGF receptor using standard methods for iodination of the ligand (14), separation of bound and free ligand and a binding assay standardized in accordance with the recommendations of the EORTC Receptor Study Group (15), and we compared our results with those obtained by other researchers with the same methodology. We also studied the association between EGF receptor and steroid receptors, and histopathological characteristics of the tumours.

Materials and Methods

Patients and tumour samples

Surgical samples were obtained from 178 patients with primary breast tumours. Thirty-five percent were premenopausal (mean age = 42.5 years, range = 26–50) and 65% postmenopausal (mean age = 63.9 years, range = 51–92).

The histopathological tumour type was known in 153 cases. The post-operative tumour size (pT) was available in 160 cases, the axillary lymph-node status (pN) in 157 cases, and the *Scarff-Bloom-Richardson* histopathological differentiation grade in 132 cases. Patient and tumour characteristics are shown in table 1.

Preparation of tissues

Samples were obtained at surgery, dissected from fat and necrotic parts and stored at −80 °C until processed. When assayed, the sample was homogenized with a Polytron apparatus (3 × 10 s bursts) in an ice-cold buffer (10 mmol/l Tris-HCl, 0.5 mmol/l dithiothreitol, 1.5 mmol/l EDTA, 1 mmol/l monothioglycerol; pH 7.4) (1+5, w/v). The homogenate was centrifuged for 1 h at 105 000 *g*. The supernatant was used for the assay of estrogen receptors and progesterone receptors and the membrane pellet was stored at −80 °C, up to four weeks, for EGF receptor determination.

Tab. 1 Patient and tumour characteristics. Numbers in parenthesis: percentage.

Patients	178	
– pre-menopausal	63	(35)
– post-menopausal	115	(65)
Tumour size	160	
– pT1 < 2 cm	51	(32)
– pT2 2– 5 cm	76	(47)
– pT3 5–10 cm	6	(4)
– pT4	27	(17)
Nodal status	157	
– Node-negative	86	(55)
– Node-positive	71	(45)
Tumour histopathology	153	
– intraductal	5	(3)
– ductal	136	(89)
– lobular	9	(6)
– others	3	(2)
Differentiation grade	132	
– G I	15	(11)
– G II	56	(42)
– G III	61	(47)

Estrogen receptor and progesterone receptor assays

Estrogen receptor (ER) and progesterone receptor (PgR) content were measured with two enzyme-immunoassays (ER-EIA and PgR-EIA Monoclonal kits, Abbott Laboratories). The assays were performed following the manufacturer's instructions. Tumours with a steroid receptor content lower than 10 fmol/mg protein were considered negative in each case.

EGF receptor assay

The stored pellets were resuspended in 2–2.5 ml of assay buffer (10 mmol/l K_2HPO_4/KH_2PO_4, 1,5 mmol/l K_2EDTA, 3 mmol/l NaN_3, 10 mmol/l monothioglycerol, glycerol (volume fraction 0.1), 50 µmol/l bacitracin; pH 7.4) by means of Polytron homogenizer (10 s burst) on ice. The homogenate was centrifuged 10 min at 800 *g* to obtain cellular membranes in the supernatant. A 100 µl aliquot was taken for membrane protein determination. To the remaining cell membrane sample assay buffer containing bovine serum albumin was added (final concentration 1 g/l).

EGF receptor was determined by a multiple point ligand binding assay. Aliquots of cell membrane preparation were incubated with ^{125}I-labelled mouse-EGF tracer (specific activity = 1110 × 10^{10} Bq/mmol) at final concentrations ranging from 2.5 to 0.07 nmol/l for 16 hours at 20 °C. The mouse EGF (Sigma) was iodinated with the lactoperoxidase – glucose oxidase reagent (Enzymobead, Bio-Rad). Non-specific binding was assessed by incubation with 250-fold excess of unlabeled mouse EGF (225 nmol/l). Receptor-bound and free ligand were separated using hydroxyapatite (DNA grade Biogel HTP, Bio-Rad). Hydroxyapatite suspension was added (final volume: 240 µl), and the assay tubes were incubated for 1 hour at room temperature. Subsequently, tubes were centrifuged for 2 min at 800 *g* and washed twice with phosphate buffer. The supernatant containing the unbound ligand was decanted and the hydroxyapatite pellet was assessed in a gamma-ray counter. EGF receptor values were calculated with *Scatchard* analysis (16), performed using a nonlinear weighted regression. EGF receptor values were expressed as fmol/mg of protein. The cut-off point was 3 fmol/mg of membrane protein, which was the lowest concentration that could be measured reliably (correlation coefficient from the *Scatchard* plot higher than 0.7).

Protein analysis

Protein concentration was determined by the method of *Lowry* et al. (17). The concentration of the membrane preparation was adjusted to the range from 0.5 to 2 g/l.

Statistical analysis

We used non-parametric tests, since the receptor data were not normally distributed: *Mann-Whitney*'s U test, χ^2 analysis for categorical variables, *Spearman* rank correlation and *Kendall* rank correlation for continuous variables and multiple regression for multivariate analysis.

Results

EGF receptor

Fifty-eight percent (103/178) of the tumours analyzed contained specific, saturable, high affinity binding sites

for EGF. Total EGF receptor content ranged from 3 to 625.1 fmol/mg of membrane protein, with a mean value of 22.2 fmol/mg (median value of 17.4 fmol/mg). *Scatchard* curves showed only one type of association site, with a median K_d of 0.462 nmol/l. Value distribution of EGF receptor in the population studied is shown in figure 1. The histogram shows a log-normal distribution.

Analysis of the steroid receptor results revealed that 70% (125/178) of the tumour biopsy samples were estrogen receptor-positive and 50% (89/178) were progesterone receptor-positive. According to estrogen/progesterone receptor phenotypes, 49% of tumours were estrogen/progesterone receptor-positive, 21% were estrogen receptor-positive/progesterone receptor-negative, 1% was estrogen receptor-negative/progesterone receptor-positive and 29% estrogen/progesterone receptor-negative.

EGF receptor was found to be positive in 48% (60/125) of estrogen receptor-positive and in 81% (43/53) of estrogen receptor-negative breast tumour biopsy samples, showing a significant inverse correlation ($\chi^2 = 16.8$, n = 178, $p = 0.00001$), and also in 48% (43/90) of progesterone receptor-positive and in 68% (60/80) of progesterone receptor-negative breast tumour biopsy samples, also with an inverse correlation ($\chi^2 = 7.6$, n = 178, $p = 0.006$). Table 2 shows the percentages of EGF receptor-positive tumours and EGF receptor content of tumours distributed according to the combined steroid receptor status.

EGF receptor content in estrogen receptor-positive tumours ($\bar{x} = 18.9$, median = 14.8 fmol/mg) was significantly lower than that in estrogen receptor-negative tumours ($\bar{x} = 53.4$, median = 18.5 fmol/mg, $p = 0.026$). Tumours with high concentrations of EGF receptor (> 100 fmol/mg) were all steroid receptor-negative. The content of EGF receptor decreased with increasing steroid receptor values (tab. 3).

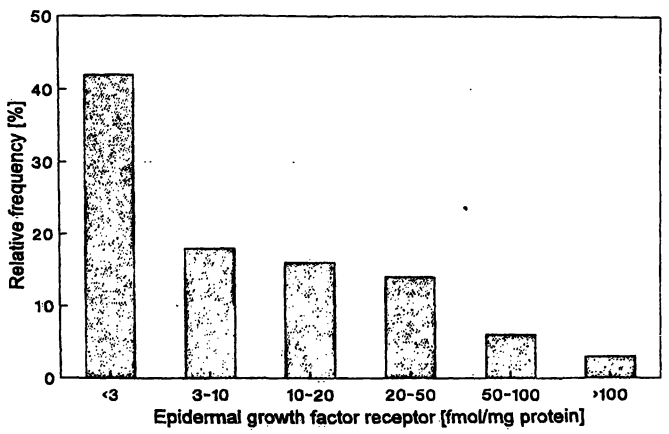

Fig. 1 Distribution of EGF receptor values in the study population. The distribution ranges from 3 to 625.1 fmol/mg membrane protein (median = 17.4).

We found a negative correlation between EGF receptor and estrogen receptor content ($r = -0.215$, $p = 0.00002$) and between EGF receptor and progesterone receptor content ($r = -0.128$, $p = 0.011$; *Kendall* rank correlation).

Multivariate analysis with EGF receptor as dependent variable and steroid receptors as independent variables showed that only estrogen receptor was independently associated with EGF receptor (estrogen receptor: $p = 0.008$, progesterone receptor: $p = 0.56$). This indicates that the association between EGF receptor and progesterone receptor depends on the strong association between progesterone receptor and estrogen receptor ($r = 0.4$, $p < 0.000001$) (*Spearman* rank correlation).

In the comparison between pre- and postmenopausal women we found differences in EGF receptor content (pre: $\bar{x} = 55.6$ fmol/mg), median = 24.2; post: $\bar{x} = 19.34$ fmol/mg, median = 13.8; $p = 0.002$). These results show an inverse relation with the values of estrogen receptor, which are higher in the postmenopausal group. No correlation was observed between EGF receptor status and age.

Histological grade

According to the *Scarff-Bloom* grading, 53% (8/15) of grade I tumours, 55% (31/67) of grade II and 64% (39/61) of grade III were EGF receptor-positive. No correlation was observed between EGF receptor and tumour grade, but considering only the tumours with high EGF receptor content (> 50 fmol/mg), 73% (8/11) were of grade III, showing a decrease of EGF receptor expression with tumour differentiation. There was a statistically significant difference between EGF receptor content in grade II tumours ($\bar{x} = 16.9$ fmol/mg), median = 9.7) and grade III tumours ($\bar{x} = 59.3$, median = 17.6) ($p = 0.027$).

Axillary nodes and histological subtypes

Regardless of the number of axillary lymph nodes invaded, there was no correlation with EGF receptor content or distribution. There was no difference between invasive, non-invasive ductal and lobular disease in the proportion of EGF receptor-positive tumours. No statistical difference was observed in terms of distribution.

Discussion

The percentage of EGF receptor-positivity in human breast cancer, measured by radioligand binding asssay,

Tab. 2 Mean EGF receptor content of EGF receptor-positive tumours distributed according to the combined steroid receptor status.

	EGF receptor-positive tumours		EGF receptor content
	%	n	(fmol/mg)
Estrogen receptor-positive/Progesterone receptor-positive	47	(41/87)	21.1*,***
Estrogen receptor-positive/Progesterone receptor-negative	50	(19/38)	14.4*,**
Estrogen receptor-negative/Progesterone receptor-positive	50	(1/2)	17.2
Estrogen receptor-negative/Progesterone receptor-negative	82	(42/51)	54.2**,***
total	58	(103/178)	

* $p < 0.04$; ** $p < 0.01$; *** $p < 0.018$

Tab. 3 Decrease of mean EGF receptor values (fmol/mg of membrane protein) with increase of estrogen receptor and progesterone receptor content (fmol/mg cytosolic protein) in breast tumours.

Steroid receptor (fmol/mg)	Estrogen receptor (fmol/mg)	Progesterone receptor (fmol/mg)
< 10	53.4	42.4
10– 50	22.1	24.4
50–100	25.4	25.0
100–250	10.9	15.5
250–500	14.0	13.6
>500	5.6	7.1

varied from 16% to 91% (8). The differences in tissue preparation and assay methodology are the most likely explanations for these variations. We found EGF receptor to be present in 58% of human breast cancers. Our positivity rate is very close to that obtained by authors who used the same methodology (18) and higher than that of many other authors (8).

Quantitative results of EGF receptor in our study ($\bar{x}$ = 33.3 fmol/mg, median = 17.4 fmol/mg) also exceed most of the values reported by other authors using radioligand binding assay which may be attributable to certain aspects of tissue preparation, the use of 800 *g* supernatant compared to higher *g*-values in most reported studies (19–21), and the EORTC methodology that we followed, such as the use of hydroxypatite to separate bound and non-bound ligand. Regarding the affinity of the receptor for its ligand, we found a K_d similar to that described in the literature for breast cancer. In this sense, we think that discrepancies regarding the presence of a single or two classes of binding sites may be due to methodological differences.

There is general agreement that the proportion of EGF receptor-positive tumours is significantly higher in estrogen receptor-negative than in estrogen receptor-positive tumours. In our study we found 81% of estrogen receptor negative tumours to be EGF receptor-positive. This concurs with *in vitro* studies of estrogen receptor-negative human breast cancer cell lines, which show a constitutive expression of EGF receptor (22). The percentages of EGF receptor-positivity in each subgroup of tumours, separated according to the steroid receptor status, are similar to those obtained previously (13, 18) by the same EGF receptor method, which is of interest with regard to the reproducibility of the assay. Furthermore, the use of different methods in the measure of steroid receptors (dextran-coated charcoal in all previous works and enzymoimmunoanalysis in our case) does not seem to influence the results (phenotypical percentages) obtained for both types of receptors, nor does it alter the conclusions.

We showed that EGF receptor-positivity and EGF receptor content were inversely associated with steroid receptor content, as reported by many authors (20, 21, 23), but we found that, performing a multivariate analysis, the negative association between progesterone receptor and EGF receptor is not independent and merely shows the strong association between progesterone receptor and estrogen receptor.

The inability to detect specific EGF binding in a large proportion of tumours analyzed (42% of the tumours in our study), has been attributed to several factors: the degradation of EGF receptor during homogenization by the action of endogenous proteases, or occupation of the receptor by endogenous EGF or EGF-like substances produced in an autocrine way (24) which impedes the receptor determination in a binding assay. This is consistent with studies performed on human cell lines for the production of tumour growth factors (26), but has recently been ruled out by other authors (26). Another possibility adduced is that since EGF receptor has been found in normal breast cells, its expression may have been lost in some tumours as a consequence of processes of malignant transformation. But this does not seem to be the case, since in our series we found a significant relationship (positive) between histopathological grade and EGF receptor-positive percentage and EGF receptor content, as reported in other studies (27).

Some immunochemical studies show that in estrogen receptor-negative/EGF receptor-positive tumours the estrogen receptor and EGF receptor expressions are mutually exclusive within individual cells (28), and it has been hypothesized that the expression of estrogen receptor may decrease and EGF receptor may increase in a particular population of cells in the later stages of malignant progression. In our series this subgroup of tumours was predominant among those without inverse correlation between estrogen and EGF receptors. We agree that the estrogen receptor-negative/EGF receptor-positive subgroup of tumours may be transitional between estrogen receptor-positive/EGF receptor-negative and estrogen receptor-negative/EGF receptor-positive tumours, indicating a tendency towards a lack of response to the therapy.

In our study, the relationship between EGF receptor and the different histopathological type of tumour was non-significant. We did not find any relationship between EGF receptor expression and tumour size, nor between EGF receptor and the invasion of axillary lymph nodes, although some authors (8) reported such relationships.

EGF receptor has been reported as a prognostic marker, especially in studies for early relapse-free survival, but the data are rather controversial. In the future, it may be important as an indicator of response to hormonal therapy and even as a target for therapeutical agents. However, this relevance is largely dependent on the reliability of its determination and different methodological approaches to measuring EGF receptor are not equivalent in terms of prognostic power. We think that the standardized methodology for EGF receptor assays, established by the EORTC group, is an easy and reproducible alternative which will expand EGF receptor determination and permit comparability of EGF receptor data between laboratories, and offers an important advance for the clincial trials involving EGF receptor.

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