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Epidermal Growth Factor Receptors in Human Breast Cancer: a Plea for Standardisation of Assay Methodology

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In a prospective study 200 primary human breast cancer specimens were analysed for epidermal growth factor receptor (EGFR) content by means of a multiple point ligand binding assay, proposed by the EORTC Receptor Study Group to be the standard EGFR assay. In 54% of the tumours the presence of saturable high affinity binding sites for epidermal growth factor could be demonstrated. The median EGFR level was 34 fmol/mg of membrane protein, the median K_d 0.50 nmd. Univariate analysis of the EGFR data stratified according to patient age, menopausal status, tumour size, axillary lymph node status, histological tumour type, tumour differentiation grade or the tumours' steroid hormone receptor status showed EGFR to be positively associated with younger age ($P = 0.03$), tumour dedifferentiation ($P = 0.04$) and steroid hormone receptor negativity ($P < 0.001$). No association between EGFR and menopausal status, tumour size, axillary lymphnode status or histological tumour type could be demonstrated.
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INTRODUCTION

In 1987 Sainsbury *et al.* [1] reported the presence of epidermal growth factor receptors (EGFR) to be an indicator of poor prognosis in human breast cancer. Since then many research groups, all employing different techniques to prepare breast tumour membrane fractions as well as techniques to assay EGFR, reported a variety of EGFR data. Subsequently, inconsistent associations between EGFR and patient age, menopausal status, tumour size, axillary lymph node involvement, histological tumour type and differentiation grade, oestrogen receptor (ER) and progesterone receptor (PgR) status, established prognostic factors in human breast cancer were also reported.

Recently the EORTC Receptor Study Group proposed the hydroxylapatite (HAP) assay to emulate as a standard technique to assay EGFR in human breast cancer biopsy specimens [2]. In a prospective study 200 breast cancer membrane preparations were analysed for EGFR in our laboratory employing this so-called HAP assay.

PATIENTS AND METHODS

Tumour biopsy specimens from 200 patients with primary breast cancer, surgically removed between May 1989 and May 1990, were analysed for EGFR content. In the same tumour biopsy specimen ER and PgR were also measured. All receptor assays were performed within 4 weeks from surgery.

The following patient and tumour characteristics were recorded: age and menopausal status at first presentation, postoperative tumour size (pT) and axillary lymph node status (pN), histological tumour type and grade. The patients were considered to be postmenopausal if the last menstruation occurred more than 1 year before the primary diagnosis. In all other cases patients were considered premenopausal. The histological differentiation grade of all invasive ductal carcinomas (not otherwise specified, NOS) was assessed by one of us (D.F.) according to the criteria provided by Scarff, Bloom and Richardson [3, 4].

ER, PgR and EGFR assays were performed according to EORTC recommendations [2, 5, 6].

Steroid hormone receptor assays

After pulverisation in the frozen state the tumour tissue was homogenised by means of a microdismembrator and dissolved in 1 ml of assay buffer. The homogenate was centrifuged for 15 min at 800 *g*, 4°C, to spin down nuclei and other coarse cell fragments. The supernatant was centrifuged for 60 min at 105 000 *g*, 4°C. The ensuing supernatant was used for ER and PgR determinations by means of ligand binding assay, utilising the dextran-coated charcoal method and multiple point technique. Cut-off values for ER and PgR were set at 10 fmol/mg of cytosolic protein. The membrane (105 000 *g*) pellets were stored at −80°C.

Epidermal growth factor receptor assay

The membrane pellets were resuspended in 1.1 ml of assay buffer by means of ultrasound bursts, on ice. A 100 µl aliquot was taken for membrane protein determination (Coomassie brilliant blue method using human serum albumin as a standard) [7]. Only those samples containing over 0.2 mg of membrane protein/ml were included in this study [6]. Eight 100 µl aliquots of each membrane preparation were incubated with Protag-125 iodinated ^{125}I-mouse-EGF (mEGF). Non-specific binding was assessed in duplicate using 1 nmol/l of ^{125}I-mEGF and a 250-fold excess of unlabelled mEGF. Receptor-bound and free ligand

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Table 1. Patient and tumour characteristics

Characteristic	*n* (%)	
Patients		
Total number	200	
Mean age (range) (in years)		61 (31–89)
Menopausal status		
Premenopausal	57 (29)	
Postmenopausal	143 (71)	
Operative treatment		
Modified radical mastectomy	113 (56)	
Breast saving surgery	59 (30)	
Simple mastectomy	4 (2)	
Tumour biopsy	24 (12)	
Tumour size		
pT_1 < 2 cm	54 (27)	
pT_2 2–5 cm	107 (53)	
pT_3 5–10 cm	17 (9)	
pT_4	22 (11)	
Tumour histology		
Ductal (NOS#)	141 (70)	
Lobular	25 (13)	
Medullary	9 (4)	
Tubular	8 (4)	
Others	17 (9)	
Nodal status		
Node negative	90 (51)	
Node positive	88 (49)	
Unknown	22	
Receptors		
ER status		
— Positive, median* (range)	152 (76)	120 (10–1500)
— Negative	48 (24)	
PgR status		
— Positive, median* (range)	135 (67)	85 (10–1500)
— Negative	65 (33)	
EGFR status		
— Positive, median† (range)	108 (54)	34 (6–360)
— Negative	92 (46)	

n = Number of patients.
*Expressed in fmol/mg of cytosolic protein.
†Expressed in fmol/mg of membrane protein.

Table 2. Relations between EGFR and patient age, menopausal status, pT, pN, histological tumour type, differentiation grade, ER and PgR

		Epidermal Growth Factor Receptor			
	n	Positive *n* (%)	P_{X2}	Mean (SD)*	P_W
Age					
<50 years	49	29 (59)	0.40	59 (50)	0.03
>50 years	151	79 (52)		47 (55)	
Menopausal status					
Premenopausal	57	34 (60)	0.31	55 (48)	0.15
Postmenopausal	143	74 (52)		47 (56)	
Tumour size					
pT_1 < 2 cm	54	28 (52)		67 (70)	
pT_2 2–5 cm	107	58 (54)	0.79	41 (45)	0.08
pT_3 5–10 cm	17	11 (65)		48 (54)	
pT_4	22	11 (50)		54 (46)	
Nodal status					
Node negative	90	50 (56)	0.56	45 (40)	0.86
Node positive	88	45 (51)		54 (54)	
Tumour histology					
Ductal (NOS)	141	74 (52)		55 (61)	
Lobular	25	13 (52)		41 (26)	
Medullary	9	7 (78)	0.65	48 (35)	0.35
Tubular	8	5 (62)		19 (12)	
Others	17	9 (53)		45 (39)	
Differentiation grade					
Grade 1	30	12 (40)		44 (33)	
Grade 2	65	30 (46)	0.04	53 (71)	0.80
Grade 3	32	22 (69)		64 (64)	
Oestrogen receptor status					
Positive	152	64 (42)	<0.001	39 (47)	0.007
Negative	48	44 (92)		66 (60)	
Progesterone receptor status					
Positive	135	61 (45)	<0.001	36 (24)	0.06
Negative	65	47 (72)		69 (73)	

*Mean of positives, expressed as fmol/mg of membrane protein.

were separated using hydroxylapatite (HAP). A human placental membrane preparation containing a fixed amount of EGFR served as a positive control for ^{125}I-mEGF-EGFR binding in each series of tumour membrane preparations. Receptor values were calculated by Scatchard analysis and expressed as fmol/mg of membrane protein [8]. All EGFR analyses resulting in interpretable Scatchard plots were regarded positive. Plots were regarded interpretable if the correlation coefficient obtained from the Scatchard plot, using a minimum of six out of the eight dose points, was found to be higher than 0.83 and aspecific binding accounted for less than 50% of total binding.

Statistics

Nonparametric test statistics were applied, since the receptor data were not normally distributed. Qualitative associations between variables were assessed by the χ^2 test for contingency tables (χ^2 denoted as χ^2, degrees of freedom as DF and P as P_{X2}). Homogeneity between groups was tested non-parametrically by means of the Wilcoxon two-sample test (Kruskall–Wallis test for multiple groups) (χ^2 denoted as $\chi^2_{W(KW)}$, degrees of freedom as DF and level of significance as $P_{W(KW)}$). Test results were regarded significant at the $P < 0.05$ level. All calculations were performed using SAS (Statistical Analysing System) statistical software [9].

RESULTS

The mean age of the 200 patients was 61 years at the time of first presentation (range 31–89 years). 71% of the patients were postmenopausal. 80% of the patients had primary tumours less than 5 cm in maximum diameter. 70% of the tumours were of the invasive ductal (NOS) histological subtype. Of these tumours 21% were well differentiated (grade 1), 46% were of intermediate differentiation grade (grade 2) and 23% were poorly differentiated tumours (grade 3). In 51% (90 out of 178 patients) of the patients axillary lymph nodes were involved at primary disease. The percentages of patients with ER-positive, respectively PgR-positive tumours were 76 and 67 (Table 1).

Of the tumours 54% (108) displayed a single class of saturable, specific, high affinity EGF binding sites, median EGFR level 34 fmol/mg of membrane protein (range 6–360), with a median K_d of 0.50 nmol/l (range 0.02–1.8).

Table 3. EGFR in human breast cancer, a literature survey

		EGFR				Prognostic factors						
Reference	*n*	Median*	Range*	Cut-off.*	% Pos	Age	Meno	pT	pN	Diff	ER	PgR
19	137	3	1–121	1	48	—	—	—	—	—	ns	ns
20	95	11	1–64	1	42	—	—	—	—	—	s	—
21	104	—	4–43	5	32	—	—	—	—	—	s	—
10	108	—	4–47	10	42	—	—	s	ns	s	s	—
1	135	—	—	10	35	ns	—	s	ns	s	s	—
22	238	6	1–137	1	54	s	—	—	—	—	s	s
23	89	4	5–26	2	57	—	—	—	s	—	s	s
24	136	27	0–275	30	46	—	—	—	—	—	s	s
25	100	40	0–200	0	22	—	—	—	—	s	s	—
13	50	—	—	10	38	—	—	—	—	s	ns	—
26	171	—	0–34	0	—	ns	—	ns	ns	s	s	s
27	225	—	—	1	43	—	—	—	ns	—	s	—
28	88		Mab		60	—	—	ns	ns	ns	s	—
14	214	1	0–317	0	93	ns	ns	ns	ns	ns	s	s
15	68	4	0–33	5	37	—	ns	—	ns	s	s	ns
11	228	—	10–187	10	35	—	—	s	—	s	s	—
29	50	(8)	1–35	1	36	—	—	—	ns	—	s	—
16	220	—	—	—	42	ns	—	ns	ns	s	s	—
12	177	4	1–102	5	25	—	—	ns	ns	ns	s	s
17	87		Mab		14	—	—	ns	ns	ns	s	ns
18	109	(47)	—	10	34	ns	ns	ns	ns	ns	s	s
30	91	(9)	1–35	1	43	—	ns	ns	ns	—	s	—
6	531	40	3–3600	0	57	—	—	—	—	—	s	s
This study	200	34	6–360	0	54	s	ns	ns	ns	s	s	s

n = number of patients on study.
Values between brackets are mean EGFR values.
%Pos = percentage of N positive for EGFR.
Meno = Menopausal status.
Mab = Monoclonal EGFR antibody.
s = statistically significant association between EGFR data and the prognostic factor indicated at the top of the column.
ns = statistically non-significant association between EGFR data and the prognostic factor indicated at the top of the column.
*Values expressed in fmol/mg of membrane protein.

The results of univariate analysis of EGFR data stratified according to patient age, menopausal status, pT, pN, histological tumour type and differentiation grade, and steroid hormone receptor status are shown in Table 2.

Percentages of EGFR positivity did not differ significantly stratified according to patient age. EGFR levels were shown to be significantly higher in women below 50 years of age than in older women, mean EGFR level 59 versus 47 fmol/mg of membrane protein ($P_W = 0.03$). No differences in either EGFR levels or positivity stratified according to menopausal status, axillary lymph node status or histological tumour type could be observed. Also, no association between the percentages of EGFR positivity and tumour size could be observed. A trend towards higher EGFR levels in smaller tumours is obvious. Moreover, the EGFR levels in tumours smaller than 2 cm in maximum diameter (pT1 tumours) are significantly higher than in larger tumours ($P_W = 0.01$). Analysis of EGFR data stratified according to the tumours' histological differentiation grade, as assessed in invasive ductal carcinomas (NOS), showed the percentages of EGFR positivity to be significantly associated with histological grade ($P = 0.04$), with a trend towards higher EGFR levels in poorly differentiated tumours.

Stratification of EGFR data according to the steroid hormone receptor status showed EGFR to be significantly associated with ER as well as with PgR. EGFR was found in 42% of ER-positive and in 92% of ER-negative breast tumours ($P_{X2} < 0.001$) as well as in 45% of PgR-positive and in 72% of PgR-negative breast tumours ($P_{X2} < 0.001$). Mean EGFR levels in ER-positive breast tumour biopsy samples were significantly lower than they were in ER-negative ones, 39 versus 66 fmol/mg of membrane protein ($P_W = 0.007$). Similarly, mean EGFR levels in PgR-positive breast tumours were lower than they were in PgR-negative ones, 36 versus 70 fmol/mg membrane protein ($P_W = 0.06$).

DISCUSSION

In the present study a ligand binding assay to measure EGFR, proposed to be the standard assay within the EORTC Receptor Study Group has been employed. Specific saturable high affinity (median K_d 0.50 nmol/l) EGFR binding sites could be demonstrated in 54% of the primary breast tumours, median EGFR level 34 fmol/mg of membrane protein. Univariate analysis of the EGFR data stratified according to conventional prognostic factors in human breast cancer (Table 2) showed EGFR to be significantly associated with patient age, the tumours' differentiation grade and the steroid hormone receptor status. No association between EGFR data and menopausal status, tumour size, axillary lymph node status or histological tumour type

could be observed. In accordance with our previous report a (compared with literature data) strikingly low percentage (2%) of double (ER as well as EGFR) negative tumours was observed [6].

A vast body of data concerning the presence of EGFR in primary human breast cancer has emerged in the literature (Table 3). As can be read from this Table the EGFR levels range from 1–360 fmol/mg membrane protein, the median EGFR levels from 1–40 fmol/mg membrane protein. In 10 out of the 22 studies in which an EGFR ligand binding assay has been employed an arbitrary cut-off level for EGFR was chosen, without methodological or clinically relevant justification. Finally, the percentage of EGFR positives ranges tremendously, from 14% to 93%.

Notwithstanding the enormous variation in EGFR data, a significant negative association between EGFR and ER has been reported in 22 out of the 24 studies. In addition nine out of 12 studies reported EGFR to be also negatively associated with PgR. Patient age has been reported to be negatively associated with EGFR in two out of seven studies. Five (out of five) studies found no association between EGFR and the patients' menopausal status. Sainsbury *et al.* reported a positive association between EGFR and tumour size [1, 10, 11], nine other groups could not find any significant association between EGFR and tumour size. In contrast, in the study presented by Bolla *et al.* [12] as well as in the present study, a significant trend towards higher EGFR levels in smaller tumours could be observed. Only one out of 15 studies reported a significant association between EGFR and axillary lymph node status. In 14 studies a tumour differentiation grade was assessed. In six of these studies tumours were graded irrespective of their histological subtype [13–18], while the Scarff, Bloom and Richardson criteria only apply to ductal carcinomas (NOS). Six, out of the eight, groups who applied the Scarff, Bloom and Richardson criteria to the appropriate tumours, reported a significant positive association between EGFR data and tumour grade.

Lack of a standardised EGFR assay probably is one of the most important causes for the variability observed. Therefore, comparison of literature data (as performed above) is precarious and moreover, probably not allowed. The authors therefore urge the need for standardisation of EGFR assays 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 tumour biology.

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Prevalence of Amplification of the Oncogenes c-*myc*, *HER2/neu*, and *int*-2 in One Thousand Human Breast Tumours: Correlation with Steroid Receptors

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The frequency of oncogene amplification described in the literature shows a large fluctuation, which could be attributed to the study of relatively small series of tumours, to selection of subgroups of patients, or, especially in retrospective studies, to selection of tumour material from the tumour-bank. To address this question, we have studied amplification of c-*myc*, *HER2/neu* and *int-2/bcl*-1 genes in a series of 1052 collected human breast tumours. The retrospective and prospective subgroups in this collected series of tumours were of equal size. c-*myc* was amplified in 17.1%, *HER2/neu* in 18.7% and *int-2/bcl*-1 in 14.1%, of all breast cancer specimens studied. In the retrospective subgroup the prevalence of amplification was 18.1% for c-*myc*; 22.6% for *HER2/neu* and 11.6% for *int-2/bcl*-1, whereas in the prospective subgroup an incidence of amplification of 16.1%, 15.1% and 16.3% for c-*myc*, *HER2/neu* and *int-2/bcl*-1, respectively was observed. *HER2/neu* amplification was negatively correlated with oestrogen receptor (ER) and progesterone receptor (PR) status ($P < 0.0001$; for both), c-*myc* amplification was more prevalent in the PR-negative subpopulation ($P < 0.05$) and *int-2/bcl*-1 amplification was positively correlated with ER status ($P < 0.001$).
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INTRODUCTION

MUTATIONS (including amplifications and deletions) of (onco-) genes have been proposed to play a role in the initiation and progression of breast cancer [1]. The mutation in the target gene may influence the expression levels or specific properties of the protein product of this gene which may be a transcription factor, a signal transducer, a growth factor or its receptor. Molecular markers will likely prove valuable in clinical decision-making. Assessment of the value of these parameters as a predictor of response to therapy or as possible point of action for new treatment modalities, is important.

In breast cancer, the prevalence of amplification of the c-*myc* gene (a nuclear phosphoprotein which presence seems to be required for proliferation while its absence may be obligatory for complete withdrawal from the cell-cycle), has been reported to range from 1–41% [2–11]. The *int*-2 gene, which encodes a protein homologous to fibroblastic growth factor (FGFs, a family involved in angiogenesis, tissue induction and cell migration) [12, 13], has been reported to be amplified between 9 and 23% [7, 14–18]. The *hst*, *int*-2 and the *bcl*-1 loci (on chromosome 11q13) are usually present in amplification units. However, there is little evidence of expression of *int*-2 [19]. There are numerous reports on the amplification of *HER2/neu* (c-*erb*B-2) gene, which encodes a transmembrane protein (185 kD) with a phosphotyrosine kinase domain and which is closely related but distinct from the epidermal growth factor (EGF-R; c-*erb*B-1) gene [20–22]. The amplification data range between 8 and 46% [23–26]. The broad range of these reported oncogene amplifications could be attributed either to the study of relatively small series of patients, patient selection (especially in retrospec-

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