Immunohistochemical assay for epidermal growth factor receptor on paraffin-embedded sections: validation against ligand-binding assay and clinical relevance in breast cancer

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Summary  Epidermal growth factor receptor (EGFR) has been the subject of much research since it was first described as a prognostic factor in breast cancer. The assay methods used and results obtained vary widely between studies. In this study 88 primary breast cancers were assessed for EGFR using a novel immunohistochemical assay performed on paraffin-embedded sections. The monoclonal antibody used was raised against purified, denatured EGFR, reacts with an epitope on the external domain and does not interfere with ligand binding. Twenty-two per cent of the tumours were EGFR positive using this assay. The results obtained were significantly correlated with those obtained by ligand-binding assay (\( r=0.621, P=0.011 \)). The concordance rate was 82.4\%(P<0.0001). The majority of discordant results could be explained by the presence of benign breast tissue and other non-malignant elements which could be seen to express EGFR on the immunohistochemical assay and were excluded from the score for this, but would be incorporated into ligand-binding assay results. The well-established inverse relationship between EGFR (as measured by this assay) and oestrogen receptor (ER) was seen (\( Q=24.9, P<0.0001 \)). In addition, in this exploratory study on a limited tumour set, EGFR was a significant adverse prognostic factor (on univariate but not multivariate analysis) for both relapse-free survival (\( P=0.02 \)) and overall survival (\( P=0.03 \)) when measured by this immunohistochemical assay, but was not significant when measured by ligand-binding assay.

Keywords: epidermal growth factor receptor; immunohistochemistry; breast cancer; paraffin-embedded sections

EGFR is a 170 kDa cell-surface receptor with an external domain containing the ligand-binding region, a short transmembrane domain and an intracellular domain containing a region with tyrosine kinase activity. It is one of an expanding group of homologous transmembrane receptors with tyrosine kinase activity which currently comprises EGFR, c-erb B-2, c-erb B-3 and recently c-erb B-4 (Carraway and Cantley, 1994; Rajkumar and Gullick, 1994). EGFR has a number of ligands, including epidermal growth factor (EGF), transforming growth factor alpha, amphiregulin, cryptot and heparin-binding EGFR. EGFR is present on a number of benign and malignant human cell lines, including some human breast cancer cell lines. In vitro, EGFR and its ligands have been implicated in malignant transformation via autocrine and paracrine growth factor pathways (Normanno et al., 1994). In vivo, EGFR is expressed in a number of human tissues, both normal and malignant. Among cancers, EGFR is most strongly expressed in squamous cell carcinomas, but it is also found in a variety of other tumours, including approximately 45% of breast adenocarcinomas (Klijn et al., 1992). A number of studies have shown it to be an adverse prognostic factor in breast cancer (Sainsbury et al., 1987; Costa et al., 1988; Harris et al., 1989; Lewis et al., 1990; Spyratos et al., 1990; Nicholson et al., 1991; Toi et al., 1991; Gasparini et al., 1992; Koenders et al., 1993; Fox et al., 1994). In contrast, the literature is consistent in reporting an inverse relationship between EGFR and ER (reviewed in Klijn et al., 1992). EGFR has also been shown to be an indicator of a poor chance of response to endocrine therapy (Nicholson et al., 1988a; Harris et al., 1989; Nicholson et al., 1994). A number of assays have been used to measure EGFR, of which the most widely applied is the ligand-binding assay (LBA) (Nicholson et al., 1988a). This method requires a relatively large amount of fresh-frozen tissue, is cumbersome to perform and cannot be applied to archival material. Other methods have been used, such as enzyme immunoassay (EIA) (Iwase et al., 1993) (which also requires frozen tissue), immunohistochemistry (predominantly on frozen sections) (Parker et al., 1984), mRNA detection methods (Coombes et al., 1990), autoradiography (Reubi and Torhorst, 1989) and EGFR-associated phosphotyrosine kinase activity (Baumet-Mahieu and Lemaire, 1989).

Ligand-binding assay is generally accepted as the 'gold standard' in EGFR measurements, though even here there is disagreement about the cut-off used to define positivity (Klijn et al., 1992). Immunohistochemical assays (IHAs) are simple to perform, can be semiquantitative and have the advantage over LBA of showing the tissue distribution of EGFR. However, there are two major problems with the IHAs described in the literature. Firstly, the majority do not work on paraffin-embedded material and so cannot be applied to archival material. Secondly, few studies have attempted to validate the assays used. In addition some anti-EGFR antibodies are raised against receptor in glycosylated form. These will potentially cross-react with blood group antigens and make interpretation of results difficult (Gerdin et al., 1992). Care must therefore be taken to ensure that the antibody used (in any assay method) is raised against epitopes on the EGFR protein and not on associated carbohydrate moieties.

We have developed an immunohistochemical assay for EGFR which works on paraffin-embedded sections and has validated this on a tumour set for which EGFR had previously been measured by ligand-binding assay. To find a clinically relevant cut-off point for this assay, analysis of the prognostic significance was determined for all possible values. The expected relationship with ER status was also assessed.

Materials and methods

Patient selection

Eighty-eight cases of previously untreated primary operable breast cancer were selected from the database at the John

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Radcliffe Hospital from 1989 to 1991. The criteria for selection were that EGFR had been previously measured by ligand-binding assay (Nicholson et al., 1988b) and that oestrogen receptor (ER) status was known. Five micron paraffin sections from a representative block of each case were used for immunostaining.

Information on patient age, tumour size, adjuvant therapy and clinical course was taken from the breast cancer patient database. The median age of these patients was 55 years (range 28–83 years). Tumours ranged in size from 1.0 to 8.0 cm (mean 2.5 cm). In six cases nodal status was not known. Of the remaining 82, 43 were node positive (52%). Sixty per cent of cases were ER positive (≥ 5 fmol mg⁻¹ protein). Of the 88 patients, 26 received no adjuvant systemic therapy, 23 received chemotherapy alone, 35 received hormone therapy alone (33 tamoxifen, two aminoglutethimide), two received both and for two this information was not available. Follow-up was at 3 month intervals, with a median length of 39 months (range 17–51 months).

**EGFR immunohistochemical assay**

The antibody used in this assay was a mouse monoclonal, subclass IgG1, raised against purified, denatured EGFR, and kindly provided by Biogenex, CA, USA (Cat. No. MU-207-UC). It reacts with a polypeptide epitope of the external domain of the molecule and does not interfere with EGFR binding. A series of preliminary experiments were conducted to optimise the staining procedure. The final conditions selected were as follows: tissue was routinely fixed in formalin and then embedded in paraffin wax blocks. Five micron sections from the blocks were dewaxed in xylene, then hydrated through graded alcohols to water. After washing in water, endogenous peroxidase activity was blocked using a 3% solution of hydrogen peroxide in distilled water for 15 min at room temperature. Slides were washed then taken to phosphate-buffered saline (PBS) at 37°C for 15 min, followed by digestion in pronase (Sigma) at 0.05% (w/v) in PBS for 15 min at 37°C. After washing in PBS, non-specific binding was blocked using normal rabbit serum (Dako) diluted 1:5 in PBS. This was drained, primary antibody (MU207) applied at a dilution of 1:10 in PBS and the slides were then incubated at room temperature overnight. Following washing in PBS, secondary antibody (biotinylated rabbit anti-mouse IgG; Dako) was added and incubated for 1 h at room temperature. Slides were washed again and streptavidin–biotin–horseradish peroxidase complex (Dako) applied and incubated for 30 min at room temperature. Slides were washed again and developed in 0.05% 3,3’-diaminobenzidine solution with hydrogen peroxide. Sections were counterstained with Mayer’s haematoxylin. Control slides of known EGFR-positive and -negative breast cancers were included in each staining run.

Slides were scored for percentage malignant cells showing membrane staining, averaged across ten high-power fields, and for overall intensity of staining on a scale of 0–3 (0 being no staining, 1 weak staining, 2 moderate staining and 3 strong staining for positive staining). The two scores were then multiplied to give the final result (possible range 0–300). A score of ≥ 35 was counted as positive (see Results). The staining and scoring for EGFR were performed without knowledge of any other data (e.g. ER, EGFR (LBA), clinical outcome). The preliminary assay optimisation experiments indicated that although staining for EGFR was heterogeneous within any one section, scores obtained by this method were similar for different sections of a tumour, and even for initial diagnostic biopsy and surgical excision specimens from the same patient.

**EGFR ligand-binding assay**

This was performed as previously described (Nicholson et al., 1988b). Results are expressed as fmol mg⁻¹ protein; a value of ≥ 20 fmol mg⁻¹ protein is the cut-off used for this assay in routine practice in Oxford.

**ER assay**

This was measured by the dextran-coated charcoal method (EORTC, 1980). Tumours with ≥ 5 fmol mg⁻¹ cytosolic protein were considered positive.

**Results**

**EGFR immunostaining**

The pattern of staining in these cases largely conformed to known EGFR distribution. In normal tissues within the sections, skin epithelial cells were positive, with the strongest staining being in the basal layer cells. In benign breast tissues, both luminal and myoepithelial cells stained, with myoepithelial cells generally stronger. Stromal fibroblasts were weakly positive in some areas; both normal and malignant. Inflammatory cells were negative, with the exception of occasional plasma cells, in which intense cytoplasmic staining was seen, and some foamy macrophages in areas of duct ectasia which showed weak membrane staining. Smooth muscle in blood vessel walls stained, as did nerve sheath cells.

Within the breast cancers, when positive, malignant epithelial cells showed clear membrane staining (Figure 1). This membrane staining was generally heterogeneous throughout the tumour. In addition, in a small number of cases there were some linear streaks of positive staining in the stroma surrounding nests of tumour cells. This was interpreted as representing remnants of normal tissue disrupted by malignant expansion. Only cases in which clear membrane staining of malignant epithelial cells was seen were counted as positive and scored. In a number of cases foci of benign breast epithelium staining positively for EGFR were found within tumours which were negative for EGFR (Figure 2).

**Definition of positivity for EGFR**

A discriminatory value for EGFR(IHA) positivity was optimised by continuous testing across the range of all possible values in relation to prognosis (relapse-free and overall survival). The value selected was that which was associated with the greatest statistical significance for the comparison between the two groups it defined. For EGFR(IHA) this gave an optimal cut-off of 35, with 22% of the cases defined as positive. This compared with 44% of cases positive for EGFR(LBA) using the conventional cut-off of ≥ 20 fmol mg⁻¹ protein.

Optimising the cut-off for the EGFR(LBA) data gave a cut-off value of approximately 80 fmol mg⁻¹, defining only 12% of cases as positive. This was too small a group on which to perform meaningful survival analysis. To allow a valid comparison between the prognostic significance of the two methods, an alternative approach was taken: a cut-off for EGFR(LBA) which defined the same proportion (22%) of cases as positive as the optimised EGFR(IHA) was found. This gave a cut-off of 38 fmol mg⁻¹.
The different cut-offs are referred to below as EGFR(LBA-20) and EGFR(LBA-38). For all analyses the optimised cut-off of 35 is used for EGFR(IHA).

Correlation with ligand-binding assay results
Of the 19.88 (22%) cases which showed positive immunostaining of malignant epithelial cells, 14.19 (74%) were also positive on ligand-binding assay (LBA-20). Of those negative on immunostaining, 44.69 (64%) were also negative on ligand-binding assay. Overall there was agreement between EGFR as assessed by immunostaining (EGFR-IHA) and on ligand-binding assay (EGFR-LBA-20) in 66% of cases (P = 0.004). On a simple linear regression plot (Figure 3), it can be seen that the majority of the cases with discordant results were negative on immunostaining but positive to a variable degree on LBA. It is probably significant that, of the 25 cases which were negative for EGFR (IHA) but positive for EGFR (LBA-20), 16 sections also contained some benign breast epithelium, and in 14 of these 16 cases the benign epithelial elements were positive for EGFR. The intensity of staining in benign breast epithelium was similar to that seen in malignant epithelium. When the optimised LBA cut-off (LBA-38) was applied, the concordance increased to 82% (P<0.0001). It can be seen from Figure 3 that the improved concordance using EGFR (LBA-38) is largely due to the loss from the relationship of cases which were IHA negative but LBA-20 positive.

Association with other parameters
No association was found between EGFR measured by either method and patient age or tumour size. Table I shows the number of EGFR-positive cases divided according to number of nodes involved for both assay methods. The expected inverse relationship with ER exists for EGFR as measured by IHA (χ² = 24.9, P < 0.0001) and for EGFR (LBA-38) (χ² = 8.3, P = 0.0071), though it is not significant for EGFR (LBA-20) (Table I). Only two tumours were positive for both EGFR (IHA) and ER.

Prognostic value
Table II shows the results of univariate analysis of prognostic factors for this set of patients. It can be seen that, while nodal status was the most powerful prognostic factor of those analysed, size ≥ 2.5 cm (P = 0.01), ER status (P = 0.05) and EGFR (IHA) (P = 0.02) were all significant

Table I Relationship between ER and EGFR

| ER    | EGFR(IHA) | EGFR(LBA-20) | EGFR(LBA-38) |
|-------|-----------|--------------|--------------|
|       | -ve       | +ve          | -ve          | +ve          | -ve         | +ve          |
| -ve   | 18        | 17           | 15           | 20           | 22          | 13           |
| +ve   | 51        | 2            | 34           | 19           | 47          | 6            |
| χ² = 24.9, P < 0.0001 | Not significant | χ² = 8.3, P = 0.0071 |

Table II Univariate analysis of prognostic factors for relapse-free (RFS) and overall (OS) survival

| Factors                  | RFS Hazard ratio | RFS p-value | OS Hazard ratio | OS p-value |
|--------------------------|------------------|-------------|-----------------|------------|
| EGFR(IHA)                | 2.38 (1.11--5.12) | 0.02        | 2.60 (1.09--6.22) | 0.03       |
| EGFR(LBA-20)             | 1.15 (0.56--2.35) | NS          | 1.09 (0.47--2.53) | NS         |
| EGFR(LBA-38)             | 1.82 (0.83--3.99) | NS          | 2.13 (0.87--5.24) | NS         |
| Age (≥ 55 years)         | 1.22 (0.60--2.50) | NS          | 1.35 (0.58--3.12) | NS         |
| Size (≥ 2.5 cm)          | 2.51 (1.17--5.37) | 0.01        | 1.41 (0.58--3.39) | NS         |
| ER                       | 0.49 (0.24--1.00) | 0.05        | 0.77 (0.33--1.79) | NS         |
| No. of nodes             |                  |             |                 |            |
| 0                        | 1.00             |             | 1.00            |            |
| 1--3                     | 2.39 (1.47--3.87) | <0.001      | 3.35 (1.83--6.13) | <0.001     |
| ≥ 4                      | 5.70 (3.52--9.24) |             | 11.21 (6.12--20.53) |            |

*Figures in brackets = 95% confidence intervals. NS, not significant.
for relapse-free survival (RFS), and EGFR (IHA) \((P = 0.03)\) was the only other significant factor for overall survival (OS). EGFR (LBA-20 or -38) was not significant in either category. Figure 4 shows the survival curves for RFS and OS stratified by EGFR(IHA) (log-rank test and Kaplan–Meier product-limit method). Of the 88 patients, 23 had died during the follow-up period (15 node positive); ten had suffered recurrence but were still alive (seven node positive) and 55 remained disease free.

In multivariate analysis, using the Cox regression model and including all of the factors significant in univariate analysis, nodal status \((P < 0.001)\) and ER status \((P < 0.01)\) were independent factors for RFS; only nodal status was an independent prognostic factor for OS \((P < 0.001)\).

**Discussion**

Since Sainsbury et al., first described EGFR as a prognostic factor in human breast cancer in 1985 (using a ligand-binding assay method), there have been a large number of studies which have measured EGFR in breast cancers by a variety of methods and with differing definitions and thus proportions of positivity. Grimaux et al. (1989) did not find EGFR (measured by LBA) to be of prognostic significance in a group of 68 node-positive cases but used a cut-off of 5 fmol mg\(^{-1}\) protein rather than 10 fmol mg\(^{-1}\), which was more widely used. Using 10 fmol mg\(^{-1}\) as a cut-off, Spyratos et al. (1990) again did not find EGFR to have prognostic significance in either node-positive or node-negative patients. Other studies have agreed with Sainsbury’s findings (e.g. Costa et al., 1988, using LBA and Lewis et al., 1990, using IHA on frozen sections).

Ligand-binding assay is the most widely applied method and is the current ‘gold standard’ for measuring EGFR. Immunohistochemical assays have the general advantages of being quick and simple to perform, requiring little material and showing the tissue distribution of the antigen concerned. Some studies have compared the results of LBA and IHA assays for EGFR. In their original study, Sainsbury et al. (1985) found that results obtained by immunostaining with the EGFR-1 antibody on frozen sections ‘correlated with’ the results of ligand-binding assay. In a more formal comparison, Toi et al. (1989) found a 94% concordance between immunostaining with the EGFR-1 antibody and results of ligand-binding assay. Using the same antibody in ovarian carcinomas, a concordance of 67% was found between the two methods (Owens et al., 1992). Using a different antibody, MAb 425, on breast cancers, no significant differences were seen when EGFR which had been measured both biochemically and immunohistochemically was independently correlated with other tumour characteristics, but this study did not directly compare the two assay results (Beckman et al., 1993). All of the above immunohistochemical studies were performed on frozen sections. A recent study has compared LBA, EIA and IHA and found reasonable agreement between the results of the three methods (72% concordance between IHA and LBA) (Iwase et al., 1993). That study concluded that EIA is the most appropriate method for use with clinical samples on the basis that it had the strongest prognostic value in the patient group examined. None of the IHAS described in these studies were conducted on paraffin-embedded sections, a procedure which has been difficult to perform successfully for EGFR. The availability of such an assay would be extremely valuable in the investigation of the biological/clinical significance of EGFR in the enormous stores of archival pathological material.

We have described and validated an immunohistochemical assay for EGFR which works on formalin-fixed, paraffin wax-embedded sections. This employs a monoclonal antibody which is raised against purified EGFR and does not cross-react with blood group antigens. This cross-reaction has been shown to be a problem with some previously described immunohistochemical assays for EGFR (Gerdin et al., 1992).

The pattern of staining produced by this assay is consistent with the known distribution of EGFR in breast tissues, both benign and malignant, and also in other normal components of these sections such as skin, nerves and smooth muscle (Damjanov et al., 1986). It has the significant advantage over LBA/EIA of showing the tissue distribution of the EGFR, eliminating the problem of possible confounding of results by expression in normal breast epithelium (and other tissue elements). Benign breast epithelium has been previously shown to express higher mean levels of EGFR than malignant breast epithelium by both LBA and immunohistochemistry on frozen material (Travers et al., 1988; Barker et al., 1989; Dittadi et al., 1993).

The proportion of tumours staining positively by this IHA is relatively low (22%). This is at the lower end of the range of positivity rates found in other studies (14–91% for a variety of assay methods, 14–65% for immunohistochemical analyses using the EGFR1 antibody on frozen tissue; Klijn et al., 1992). Our positivity rate may be within the expected variability range found when comparing results across several often relatively small studies. Alternatively, it may relate to the assay method in which antigen retrieval in the form of protease digestion is essential. The duration of the protocol is limited by the need to preserve tissue morphology. It is possible therefore that the sensitivity of the assay is reduced relative to other methods as one may be unable to ‘retrieve’ all antigen present while still preserving tissue morphology.

Measurement of EGFR using this immunohistochemical assay shows a positive, but by no means perfect, correlation with the ligand-binding assay. There are good reasons for arguing that LBA, or any of the other methods which use homogenates of tissue, are not the most appropriate methods for assessing EGFR in clinical material. The majority of discordant results are those which are negative by immunostaining but positive by LBA. The majority of these have positive-staining benign breast epithelium. While these paraffin sections are not contiguous with the portions of tumour used in the LBA, it does provide...
The data on comparability are thus very encouraging, indicating that the IHA is likely to be at least as useful as the LBA for clinical assessment of EGFR. The statistically stronger relationships with EGFR(IHA) may be due to the exclusion by this approach of EGFR-expressing normal benign tissue, which would be expected to be biologically less relevant (or entirely irrelevant) in relation to disease progression. Confirmation of the prognostic significance of EGFR(IHA) would obviously require a much larger patient group. Such a study is planned for the near future.

In conclusion, we have developed and validated an immunohistochemical assay for EGFR which is quick and simple to perform, requires small amounts of tissue and can be applied to paraaffin-embedded sections and thus to archival material.

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