Virchows Archiv A Pathol Anat (1991) 419:171–175

Virchows Archiv A
Pathological Anatomy and Histopathology

Immunohistochemical distribution patterns of epidermal growth factor receptor in malignant mesothelioma and non-neoplastic mesothelium

Marc Ramael, Kurt Segers, Corinne Buysse, Jan Van den Bossche, and Eric Van Marck

Laboratory of Pathology, University Hospital Antwerp, Wilrijkstraat 10, B-2650 Edegem, Belgium

Received April 3, 1991 / Accepted June 18, 1991

Summary. An immunohistochemical study of the epidermal growth factor (EGF) receptor in non-neoplastic pleural mesothelium (35 cases) and in human malignant mesothelioma (36 cases) was made, using a murine monoclonal antibody OM-11-951. All malignant mesotheliomas and non-neoplastic pleural biopsies exhibited a strong cytoplasmic immunoreactivity in mesothelial cells. Nuclear immunoreactivity was detected in mesothelial cells of all specimens of both malignant and non-neoplastic pleura. No statistically significant differences were found between malignant mesothelioma and non-neoplastic pleural mesothelium. There were differences, between the three subtypes of mesothelioma, in the number of cells that exhibited nuclear staining. Statistically significant differences were noted between the epithelial subtype and the mesenchymal subtype ($P < 0.005$), epithelial subtype versus the mixed cell type ($P < 0.005$) and between the mesenchymal component of the mixed cell type and the mesenchymal type ($p < 0.0005$). We conclude that there is strong expression of EGF receptor in both malignant mesothelioma and in non-neoplastic pleural mesothelium. Different staining patterns are seen when comparing the different subtypes of mesotheliomas with each other. EGF receptor expression cannot be used to distinguish between malignant and benign mesothelium.

Key words: Epidermal growth factor receptor – Mesothelioma – Pleura – Nuclear immunoreactivity – Immunohistochemistry

Introduction

Several oncogenes and their products have been detected in human tumours and there is increasing evidence that oncogenes may be involved in different stages of the multi-step carcinogenesis process (Land et al. 1983). Epidermal growth factor (EGF) is a polypeptide with potent mitogenic activity that stimulates proliferation of a variety of tissues through interaction with its receptor, a transmembrane protein with a molecular weight of 170 kDa (Carpenter and Zendegui 1986). In response to EGF, the receptor kinase is capable of autophosphorylation and phosphorylates intracellular substrates (Carpenter 1983; Nakamura et al. 1983) after which the mitogenic signal is generated. The EGF receptor has certain structural similarities with the avian erythroblastosis virus v-*erb*-B transforming protein (Downward et al. 1984). EGF receptors have been identified in some gliomas (Reifenberger et al. 1989), gastrointestinal tumours (Koretz et al. 1990; Sugiyama et al. 1989), bladder tumours (Neal et al. 1985), breast tumours (Sainsbury et al. 1984), and in sarcomas (Gusterson et al. 1985).

The aim of the present study was to investigate the distribution patterns of EGF receptor in epithelial, mesenchymal and mixed mesotheliomas, compared to its distribution pattern in non-neoplastic mesothelium.

Materials and methods

A total of 36 paraffin-embedded mesothelioma tissue specimens comprising 25 epithelial mesotheliomas, 8 mixed mesotheliomas and 3 mesenchymal mesotheliomas were included in the study together with 35 paraffin-embedded pleural tissue specimens or pleural exudates with non-neoplastic mesothelium. Patients with a previous history or suspicion of malignancy were not included in the latter group. All samples were fixed in alcoholic formalin acetic acid solution. After sectioning and haematoxylin and eosin staining, periodic acid-Schiff (PAS), PAS after diastase digestion, alcian blue, and alcian blue after hyaluronidase digestion staining was also performed. Immunohistochemical staining for carcinoembryonic antigen (CEA), cytokeratin, vimentin and epithelial membrane antigen (EMA) was also performed, employing standard PAP-immunohistochemical procedures.

Paraffin sections (5 μm thick) were cut and dewaxed in xylene followed by rehydration in decreasing ethanol series, water and phosphate-buffered saline (PBS), pH 7.4. Slides were immersed in methanol for 30 min to block the endogenous peroxidase and incubated with non-immune rabbit serum (diluted 1:20 in PBS) for 20 min to block the non-specific Fc receptor activity in tissue. The sections were consecutively treated for 30 min with:

Offprint requests to: M. Ramael

1. Anti-EGF receptor mouse IgG_1 monoclonal antibody OM-11-951 (Cambridge Research Biochemicals, Cambridge, UK) diluted 1:1000 in PBS, pH 7.4, supplemented with 1% bovine serum albumin at room temperature. The mouse monoclonal anti-EGF receptor antibody OM-11-951 (isotype IgG_1) is directed against a synthetic dodecapeptide derived from the extracellular domain of the human EGF receptor protein sequence. There is no sequence similarity with aligned regions from the human *pp60$^{c\text{-}src}$* sequences (Parker et al. 1984).
2. Polyclonal rabbit anti-mouse IgG_1 antibody (Dako, Glostrup, Denmark), diluted 1:20 in PBS with 1% normal human serum.
3. Horseradish mouse peroxidase-mouse polyclonal anti-horseradish-peroxidase complex diluted 1:250 in PBS (Dako). Steps 2 and 3 were repeated for 10 min each to enhance the reaction.

Peroxidase was revealed by incubating the sections with a solution containing 10 mg of 3,3′ diaminobenzidinetetrahydrochloride in 20 ml TRIS buffer, pH 7.6, containing 2% hydrogen peroxide. The specificity of the immunohistochemical reactions was controlled by omitting the first antibody and by substituting the anti-EGF antibody for an unrelated monoclonal antibody in the same concentration (monoclonal mouse anti-human IgM, isotype IgG_1, Dako). Sections of oesophageal squamous epithelium and prostate were used as positive controls for each series of stainings. Counterstaining was with Mayer's haematoxylin for 40 s. The chi-square test was applied for statistical analysis.

Results

Thirty-six specimens from patients with a malignant mesothelioma and 35 specimens of non-neoplastic mesothelium were studied.

The mean age of the mesothelioma patients was 60.8 years (range 39–80 years) with a male- to female ratio of 6.2:1. Of the mesotheliomas 25 were epithelial, 3 mesenchymal and 8 were of the mixed cell type. All mesotheliomas exhibited alcian blue hyaluronidase sensitive positivity and, immunohistochemically, all were vimentin, EMA and cytokeratin positive, and CEA negative.

Positive control sections showed moderate to strong cytoplasmic immunoreactivity for EGF receptor. No nuclear staining was seen in these sections.

The results of the immunohistochemical staining for the EGF receptor are summarized in Tables 1 and 2. In mesotheliomas, staining of nuclei and cytoplasm was found in all cases. Not all tumour cells were positive. There was a significant difference between the different mesothelioma types with respect to nuclear staining. Nuclear staining was in a fine granular diffuse fashion. Of the epithelial mesotheliomas 64% exhibited nuclear immunoreactivity in more than 75% of all nuclei (Fig. 1A, B). All mesenchymal mesotheliomas exhibited nuclear immunoreactivity in 25–50% of all nuclei (Fig. 2). Mixed cell type mesotheliomas showed a broad range of nuclear immunoreactivity (Fig. 3A). However, the epithelial and the mesenchymal component showed a differential nuclear staining. In the epithelial component 63% of cells showed immunoreactivity in 50–75% of the nuclei (Fig. 3B), while 88% of cells of the mesenchymal component displayed immunoreactivity in less than 25% of the nuclei (Fig. 3C). In nearly all mesotheliomas cytoplasmic staining was present in more than 75% of the cells.

More than 75% of non-neoplastic mesothelial cells showed an intense, homogeneous cytoplasmic immunoreactivity for EGF receptor (Fig. 4). Nuclear staining of mesothelial cells, as a diffuse, fine granular pattern, was found in mesothelium of non-neoplastic pleura. In 57% of cases of non-neoplastic mesothelium more than 75% of all mesothelial cell nuclei were immunoreactive. There was only minimal background staining. Weak staining of nerves and fibroblasts occurred. Muscle and adipocytes showed heavy staining.

There was no immunoreactivity in negative control sections. Statistical analysis revealed no significant differences in cytoplasmic and nuclear staining between non-neoplastic mesothelium and mesotheliomas. There was, however, a statistically significant difference in nuclear staining between epithelial and mesenchymal mesothelioma ($P<0.0005$), and between epithelial and mixed cell type ($P<0.005$). Within the mesotheliomas of mixed cell type, there was a statistically significant difference between the epithelial and mesenchymal components ($P<0.001$). No difference was seen between the epithelial component of a mixed cell type and an epitheli-

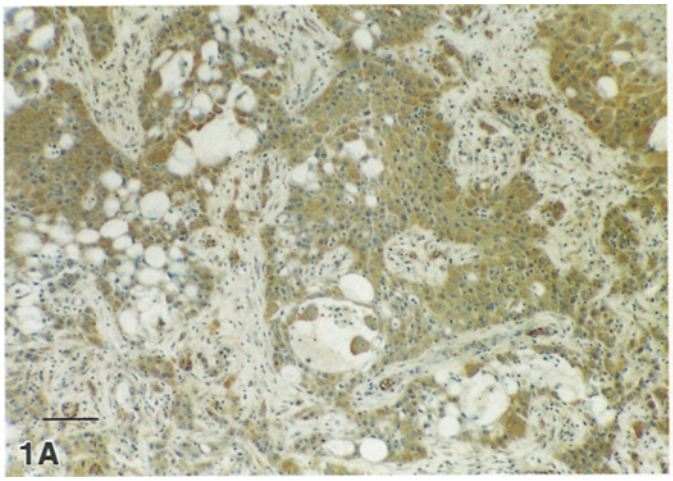
Table 1. Cytoplasmic immunoreactivity for the epidermal growth factor receptor. Comparison between the various subtypes of mesothelioma and non-neoplastic mesothelium

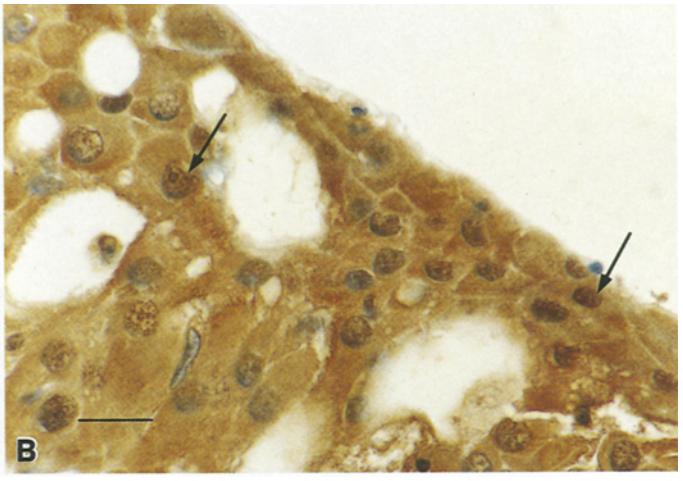
Positive cells (%)	<25	25–50	50–75	>75
Mesothelioma type				
Epithelial	1 (4%)			24 (96%)
Mesenchymal				3 (100%)
Mixed	1 (12%)			7 (88%)
Epithelial component	1 (12%)			7 (88%)
Mesenchymal component	1 (12%)			7 (88%)
Non-neoplastic mesothelium			5 (14%)	30 (86%)

Table 2. Nuclear immunoreactivity for epidermal growth factor receptor: comparison between the various subtypes of mesothelioma and non-neoplastic mesothelium

Positive cells (%)	<25	25–50	50–75	>75
Mesothelioma type				
Epithelial	2 (8%)	1 (4%)	6 (24%)	16 (64%)
Mesenchymal		3 (100%)		
Mixed		3 (37%)	4 (50%)	1 (13%)
Epithelial component	1 (12%)		5 (63%)	2 (25%)
Mesenchymal component	7 (88%)	1 (12%)		
Non-neoplastic mesothelium	5 (14%)	10 (29%)		20 (57%)

Percentages of cases in each group shown in parentheses





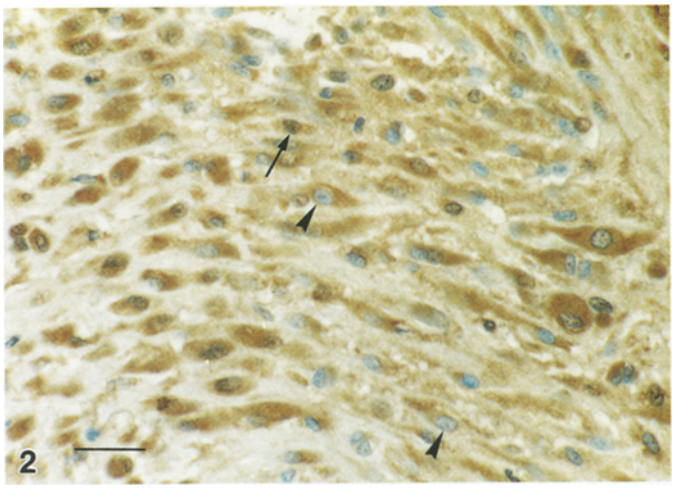


Fig. 1. **A** Epithelial malignant mesothelioma. Strands of tumour cells embedded in stroma containing pre-existing adipocytes. Cytoplasmic immunoreactivity for EGF receptor in almost all neoplastic cells. *Bar* = 400 μm. × 25. **B** Same epithelial mesothelioma as in **A**. Most of the cells have nuclei (*arrows*) and cytoplasm immunoreactive for EGF receptor. *Bar* = 100 μm. × 130

Fig. 2. Mesenchymal malignant mesothelioma. Most of the neoplastic cells are immunoreactive for EGF receptor. Some nuclei show immunoreactivity (*arrow*); others are negative (*arrowheads*). *Bar* = 150 μm. × 80

al mesothelioma ($P > 0.05$). In contrast there was a statistically significant difference between the mesenchymal component of a mixed cell type and the mesenchymal mesotheliomas ($P < 0.0005$).

Discussion

EGF receptor is found in many normal and neoplastic tissues. A correlation between EGF receptor expression, tumour invasion and the presence of EGF has been described in gastric carcinoma (Sugiyama et al. 1989). Invasive and poorly differentiated bladder tumours stain more intensely for EGF receptor (Neal et al. 1985). An association between EGF receptor positivity and survival has been shown for breast cancer (Sainsbury et al. 1984). The expression of EGF receptor can be valuable in the diagnosis of lung tumours (Berger et al. 1987). Our studies did not reveal a significant difference in reactivity for EGF receptor between non-neoplastic mesothelium and mesothelioma. Both showed a strong cytoplasmic staining. More than 75% of all cells were immunoreactive for EGF receptor. This contrasts with recently published results (Dazzi et al. 1990) where the authors observed considerably more variation in cytoplasmic staining intensity. Nuclear staining was not detected in mesotheliomas. Data on non-neoplastic mesothelium are not available in the literature. In our series, nuclear staining was present in nearly all cases of mesothelioma and in non-neoplastic mesothelium. Nuclear staining patterns did not differ between non-neoplastic and neoplastic mesothelium, though statistical differences were noted between the epithelial and the mixed cell type mesotheliomas. The divergence between our results and the results of the study of Dazzi et al. (1990) may be due to the different fixation procedures and the difference in anti-EGF receptor antibody. Another possibility is that our method is more sensitive in tracing small amounts of EGF receptor immunoreactivity in the nucleus.

Three patterns of immunoreactivity for the EGF receptor are described in the literature: cell surface expression as described for the A431 epidermoid carcinoma cell line (Waterfield et al. 1982), diffuse cytoplasmic staining as observed in several normal tissues (Gusterson et al. 1984) and nuclear immunoreactivity as described in epithelial tissues (Gusterson et al. 1984), adrenocortical carcinoma (Kamio et al. 1990) and sarcomas (Gusterson et al. 1985). Cytoplasmic staining for EGF receptor is due to EGF receptor production and to internalization of the EGF receptor complex. The EGF receptor complex is first located on the cell surface and is then transferred to the endosomal compartments (Carpenter and Cohen 1976). Some EGF receptors are re-utilized

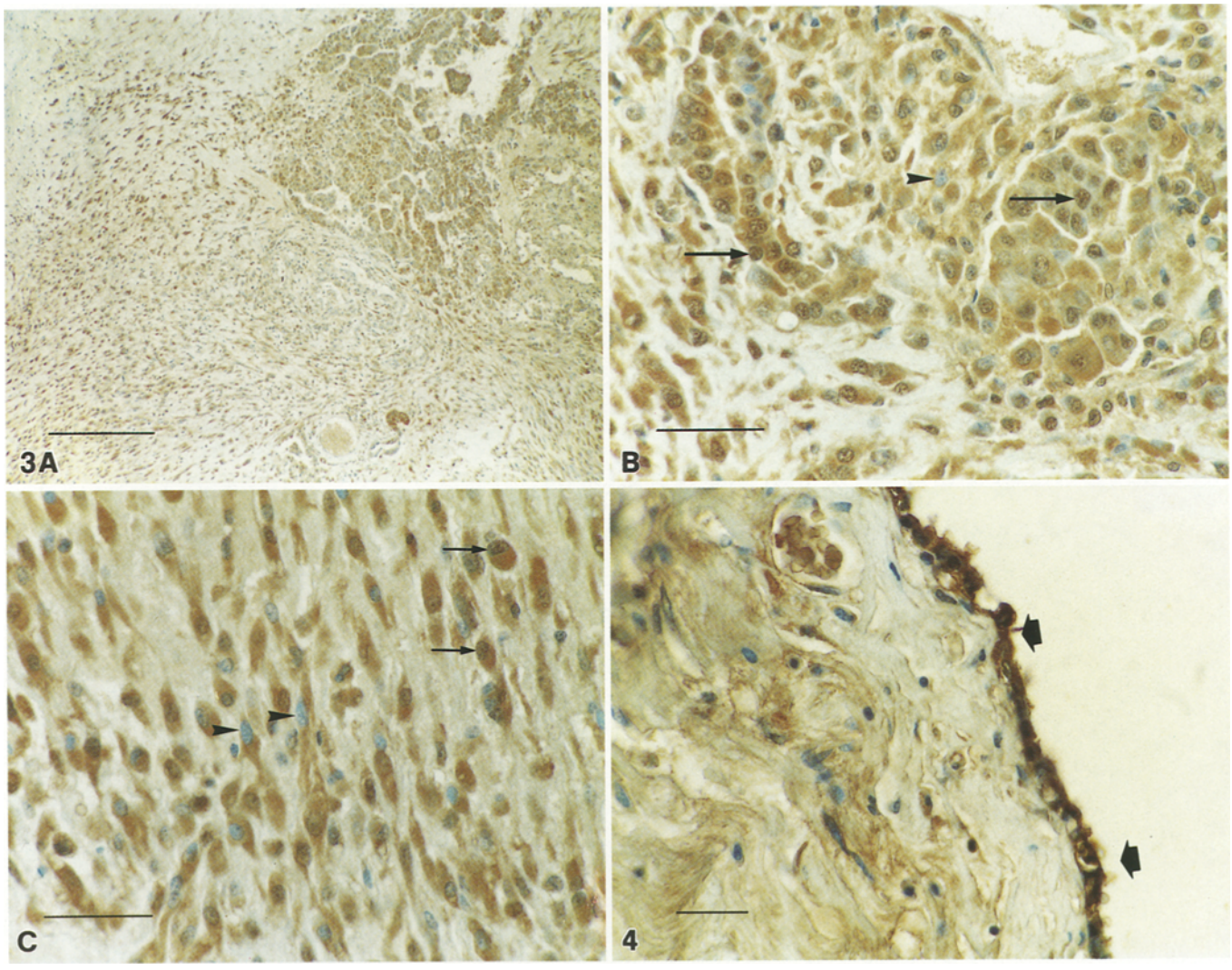


Fig. 3. **A** Mixed malignant mesothelioma; the epithelial component is to the right, the mesenchymal to the left. Cytoplasmic immunoreactivity for EGF receptor is seen in most of the neoplastic cells. *Bar* = 100 μm. × 20. **B** Higher magnification of the epithelial component of the same mixed type mesothelioma as in **A**. Most nuclei (*arrows*) and the cytoplasm of the neoplastic cells are immunoreactive for EGF receptor. Some nuclei are negative (*arrowhead*). *Bar* = 200 μm. × 100. **C** Higher magnification of the mesenchymal component of the same mixed type mesothelioma as in **A**. Most cytoplasms are immunoreactive for EGF receptor. Some nuclei (*arrows*) are immunoreactive, while others are not (*arrowheads*). *Bar* = 200 μm. × 100

Fig. 4. Non-neoplastic pleural mesothelium with strong immunoreactivity for EGF receptor (*arrows*). *Bar* = 100 μm. × 130

after internalization (Dunn et al. 1984). Others are degraded in lysosomes. However, internalized complexes still have the capacity to autophosphorylate and to phosphorylate plasma membrane-inaccessible substrates (Cohen and Fava 1985). These authors hypothesize that vesicles could fuse with the nuclear membrane and would permit direct receptor-kinase-mediated phosphorylation of a regulatory nuclear protein. Experiments with ^{125}I-EGF performed in both an EGF-non-responsive mutant fibroblast line 3T3-NR6 and in the same cell line reconstituted with active EGF receptor demonstrated not only predominantly intracellular localization of the receptor but also yielded evidence for the existence of a growth factor/nuclear signalling mechanism depending on the nuclear acquisition of EGF binding activity from the plasma membrane (Jiang and Schindler 1990). This could explain the nuclear staining in some epithelial tissues, sarcomas and in the mesothelial tissues described in our study.

Rakowicz et al. (1986) described binding of EGF to chromatin, probably via receptor-like molecules. This finding is corroborated by the fact that binding of EGF to the chromatin could be prevented by specific monoclonal antibodies directed against the extracellular part of the EGF receptor. It is tempting to speculate that the differences in nuclear EGF receptor staining between the various mesothelioma subtypes reflects a different sensitivity of the nucleus to EGF. It is possible that nuclear EGF-binding molecules are acting as modulators of gene transcription. Binding of growth factors such as EGF, platelet derived growth factor and insulin to DNAse-II-sensitive regions of chromatin has been de-

scribed (Rakowicz et al. 1986). After binding the DNAse II sensitivity changed, indicating specific binding of these growth factors.

EGF should not only be considered as a mitogen; it can also influence cellular differentiation in epidermal keratinocytes, for example (Green 1977). Cultured rat pituitary cells treated with EGF increased synthesis of prolactin while growth hormone synthesis was inhibited. The isolated nuclei of these pituitary cells showed an increased capacity to bind RNA polymerase in initiation site complexes (Johnson et al. 1980).

The differences in nuclear staining for EGF receptor between the various mesothelioma subtypes could be a reflection of different transcriptional activity and may represent the expression of differences in gene activation. It is not necessarily linked to mitosis but could be a differentiation related phenomenon. This requires further investigation.

We conclude that EGF receptor expression is not of diagnostic value for discriminating between non-neoplastic and malignant mesothelium. Both exhibit strong cytoplasmic reactivity. Between subgroups of mesotheliomas there are significant differences only in nuclear staining intensities. The reason remains obscure and may be elucidated when more information is available on the internal pathway, function and fate of the internalized EGF receptor.

References

Berger M, Gullick W, Greenfield C (1987) Epidermal growth factor receptors in lung tumours. J Pathol 152:297–307

Carpenter G (1983) The biochemistry and physiology of the receptor kinase for epidermal growth factor. Mol Cell Endocrinol 31:1–19

Carpenter G, Cohen S (1976) ^{125}I labeled human epidermal growth factor: binding, internalization and degradation in human fibroblasts. J Cell Biol 71:159–171

Carpenter G, Zendegui J (1986) Epidermal growth factor, its receptor and related proteins. Exp Cell Res 164:1–10

Cohen S, Fava R (1985) Internalization of functional epidermal growth factor: receptor/kinase complexes in A431 cells. J Biol Chem 260:12351–12358

Dazzi H, Hasleton P, Thatcher N (1990) Malignant pleural mesothelioma and epidermal growth factor receptor (EGF-R). Br J Cancer 61:924–926

Downward J, Yarden Y, Mayes E (1984) Close similarity of epidermal growth factor receptor and v erb-B oncogene protein sequences. Nature 307:521–526

Dunn W, Hubbard A (1984) Receptor mediated endocytosis of epidermal growth factor by hepatocytes in the perfused rat liver: receptor and ligand dynamics. J Cell Biol 98:2148–2159

Green H (1977) Terminal differentiation of cultured human epidermal cells. Cell 11:405–415

Gusterson B, Cowley G, Smith H, Bradford O (1984) Cellular localization of epidermal growth factor receptor. Cell Biol Int Rep 8:649–658

Gusterson B, Cowley G, McIlhinney J (1985) Evidence for increased epidermal growth factor receptors in human sarcomas. Int J Cancer 36:689–693

Jiang L, Schindler M (1990) Nucleocytoplasmic transport is enhanced concomitant with nuclear accumulation of EGF binding activity in both 3T3-1 and EGF receptor reconstituted NR-6 fibroblasts. J Cell Biol 110:559–568

Johnson L, Baxter J, Vlodansky E, Gospodarowicz H (1980) Epidermal growth factor and expression of specific genes: effects on cultured rat pituitary cells are dissociable from the mitogenic response. Proc Natl Acad Sci USA 77:394–398

Kamio T, Shigematsu K, Sou H, Kawai K, Tsuchiyama H (1990) Immunohistochemical expression of epidermal growth factor receptors in human adrenocortical carcinoma. Hum Pathol 21:277–282

Koretz K, Schlag P, Möller P (1990) Expression of epidermal growth factor receptor in normal colorectal mucosa, adenoma and carcinoma. Virchows Arch [A] 416:343–349

Land H, Parada L, Weinberg R (1983) Cellular oncogenes and multistep carcinogenesis. Science 222:771–778

Nakamura K, Martinez D, Weber M (1983) Tyrosine phosphorylation of specific proteins after mitogen stimulation of chicken embryo fibroblasts. Mol Cell Biol 3:380–390

Neal D, Marsh C, Bennett M (1985) Epidermal growth factor receptors in human bladder cancer: comparison of invasive and superficial tumours. Lancet II:366–368

Parker P, Young S, Gullick W (1984) Monoclonal antibodies against the human epidermal growth factor receptor from A431 cells. J Biol Chem 259:9906–9912

Rakowicz E, Ulrich R, Herlyn M, Koprowski H (1986) Chromatin binding of epidermal growth factor, nerve growth factor and platelet derived growth factor in cells bearing the appropriate surface receptors. Proc Natl Acad Sci USA 83:3728–3732

Reifenberger G, Prior R, Deckert M, Wechsler W (1989) Epidermal growth factor receptor expression in human tumours of the nervous system. Virchows Arch [A] 414:147–155

Sainsbury J, Farndon J, Sherbet G, Harris A (1984) Epidermal growth factor receptors and oestrogen receptors in human breast cancer. Breast Cancer Res Treat 44:3348–3353

Sugiyama K, Yosemura Y, Miyazaki I (1989) Immunohistochemical study of epidermal growth factor and epidermal growth factor receptor in gastric carcinoma. Cancer 63:1557–1561

Waterfield M, Mayes L, Stroobant P, Bennet P, Young S, Goodfellow P, Banting P, Ozanne B (1982) A monoclonal antibody to the human epidermal growth factor receptor. J Cell Biochem 20:149–161