EGF RECEPTOR IN BREAST CANCER: ITS QUANTIFICATION AND ROLE IN INVASION

KEVIN WILLIAM ROBERTSON

MB ChB, FRCS

A thesis submitted to the University of Glasgow

for the Degree of Doctor of Medicine

Dept. of Surgery (Royal Infirmary)

University of Glasgow

ABSTRACT

EGF receptor was first implicated in the prognosis of breast cancer patients in 1987. It has since been extensively studied, both in terms of its clinical significance and its role in tumour biology. Despite this, the mechanism of its prognostic influence remains unclear, as is its relationship with established prognostic indicators. Inaccuracies in the methods used to measure receptor expression may, in part, be responsible. This thesis addresses this issue, and then broaches the possibility that receptor mediated tumour cell invasion might account for its effect on prognosis.

Receptor measurement using a quantitative radioimmunohistochemical assay (Rihc) was compared with the most commonly used conventional methods. Using Rihc, receptor could be measured in 92% of tumours, as opposed to 38% and 42%, with ligand binding (Lb) and conventional immunohistochemistry (Cihc), respectively. The conventional methods, categorising tumours as positive or negative, showed a high level of correlation ($p=0.0006$) but for 26% of the tumours did not concur. This level of disparity may account for some of the confusion over the role of EGF receptor in breast cancer. Rihc was compared with the other methods using a Spearman rank analysis. Limiting this analysis to tumours receptor positive using the conventional technique, Rihc and Cihc had a better correlation ($p<0.0005$), than Rihc and Lb ($p=0.702$). Despite the correlation between the immunohistochemical methods, Cihc failed to detect receptor in 52% of the tumours, a group in which receptor expression varied by 10 fold using Rihc. Overall, Rihc proved more sensitive and more accurate.

In total, 203 breast cancers were analysed using Rihc. The vast majority (98%) had levels of receptor below those in normal (reduction mammoplasty) breast. In keeping with the consensus, there was a strong inverse correlation between EGF receptor and oestrogen receptor ($p<0.0005$). There was also a direct association with poorer histological grade ($p=0.005$), but none to either T-stage ($p=0.392$) or nodal status ($p=0.074$). Additionally, the accuracy of Rihc allowed identification of an inverse correlation between the levels of oestrogen and EGF receptor (rank analysis $p=0.032$), and also a direct correlation with maximal tumour size, in millimetres (rank analysis $p=0.049$). To assess the relationship with outcome, tumours were divided into groups determined by receptor expression. Using a univariate analysis, EGF receptor predicted death (from all causes) and also disease free survival; tumours studied in 2 groups ($p=0.0429$, $p=0.0446$,

respectively), 3 groups ($p=0.0047$, $p=0.0072$, respectively), and 4 groups ($p=0.0009$, $p=0.0013$, respectively). However, in multivariate analysis, oestrogen receptor (relative risk 4.7, 95%CI 2.5-8.7), then nodal status (relative risk 3.0, 95%CI 1.6-5.7), followed by tumour size (TNM) (relative risk 1.8, 95%CI 1.1-2.8), were included in the outcome model for all deaths, and EGF receptor was the least significant predictor of outcome.

It has been hypothesised that EGF receptor signalling promotes tumour cell motility and invasion. A novel invasion assay, using the basement membrane substitute Matrigel, was developed and applied to breast cancer cell lines expressing a range of receptor densities. Invasion, into the Matrigel layer, was promoted by EGF and ascertained using confocal microscopy coupled to image analysis. The MDA-MB-231 cell line invaded up to 30μm, with a clear dose response curve ($p=0.0005$). Invasion, also evident for the MCF7 Adriamycin resistant and BT20 cell lines, did not simply reflect proliferation. It occurred more frequently in the cell lines expressing more EGF receptor, but this relationship was not absolute, indicating that other factors were important. Monoclonal antibody, ICR16, directed to the EGF receptor and inhibiting EGF binding abrogated invasion, as did the tyrosine kinase inhibitor, 4,5-Dianilinophthalimide (DAPH). These results indicated that breast tumour cell invasion was, at least partly, modulated by EGF receptor.

ACKNOWLEDGEMENTS

I was introduced to this fascinating area of research by Professors Peter Stanton and Tim Cooke. I am grateful to both for the level of support and encouragement they provided throughout my research and subsequent compilation of this thesis. My gratitude extends also to Dr Bradford Ozanne and the members of his research group at the Beatson Institute for Cancer Research at Garscube. It was a privilege to have had the opportunity to perform this research under the auspices of these individuals.

At a technical level, the second and third parts of this thesis were supported greatly by the efforts of Dr Jonathon Reeves both for his guidance of my scientific endeavours and for his help establishing the breast cancer database. Gerard Smith, also of the Department of Surgery at Glasgow Royal Infirmary, deserves my gratitude for performing most of the radioimmunohistochemical work. Similarly, Dr James Going in the Department of Pathology spent many hours retrospectively grading tumour sections and performing immunocytochemical studies, work for which I am very grateful.

I would also like to acknowledge my debt to the Scottish Hospital Endowments Research Trust. Without the benefit of their SHERT/Cruden Medical Research Scholarship (955 130) the period of study would have been considerably more arduous.

Finally, I would like to thank Doctors K Rodger and J Jackson, their support and encouragement, though less technical, was no less valuable. Mention should also go to my parents, they dutifully 'phoned weekly for 4 years asking if I had finished yet!

STATEMENT

To the best of my knowledge and belief this work contains no material previously published or written by another person, except where due reference is made in the text.

TABLE OF CONTENTS

CONTENTS IN DETAIL

FIGURES

TABLES

PART 1

HISTORICAL REVIEW
AND
BACKGROUND INFORMATION

Chapter 1:
Basic science of the epidermal growth factor receptor

Section i: EGF receptor and the type I tyrosine kinase receptors

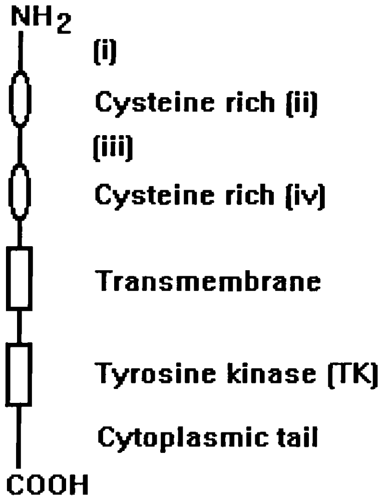
From the earliest days of tissue culture, there has been considerable interest in the mechanisms regulating the proliferation of cells. Early work indicated that specific proteins, called growth factors, interacted with receptors stimulating cell division. The discovery, in the early 1980's, of retroviral oncogenes, their cellular equivalents, and the subsequent realisation that these genes had extensive homology with those coding for growth factor receptors, intensified this interest. Consequent studies of these molecules, and their role in cell biology, have lead us to begin to unravel the functional nature of the molecular lesions which give rise to cancer.

In the 1930's, the causative agent of avian erythroblastosis and fibrosarcoma was identified, but not until much later was it realised that this was the retrovirus and oncogene, *erb*-B. Subsequently, this gene was found to exhibit extensive homology with the human EGF receptor gene. The resultant interest in the type I tyrosine kinase receptors, of which the epidermal growth factor receptor is the paradigm, means these are perhaps the most extensively studied growth factor receptors.

EGF receptor, or c-*erb*B-1, was the first identified member of the type I growth factor receptor family; the others are c-*erb*B-2, c-*erb*B-3 and c-*erb*B-4. All are cell surface glycoprotein receptors that signal via very highly conserved tyrosine kinases. All have been associated with anomalous expression in a variety of human tumours (reviewed in Prigent 1992).

Purification of EGF, one of the EGF receptor ligands, made possible the isolation of the latter from the squamous carcinoma cell line, A431, in which it is hugely overexpressed (Wrann 1979). Thereafter, the receptor's cDNA sequence (Xu 1984, Downward 1984, Lin 1984 and Ullrich 1984) and then its genomic structure (Haley 1987) were determined. Using this information, low stringency probing of human cDNA libraries with both sequences from the EGF receptor gene and its avian viral analogue, v-*erb*B, allowed identification of the other members of the type I growth factor receptor family.

Figure 1.1 : Amino acid homology in the type I tyrosine kinase receptor family.



	% amino acid homology				
	i	ii	iii	iv	TK
EGFR / c-*erb*B-2	42	46	45	41	82
EGFR / c-*erb*B-3	40	46	46	50	60
c-*erb*B-2 / c-*erb*B-3	40	45	44	41	62
EGFR / c-*erb*B-4	46	51	42	45	79
c-*erb*B-2 / c-*erb*B-4	46	46	39	34	77
c-*erb*B-3 / c-*erb*B-4	50	67	60	49	63

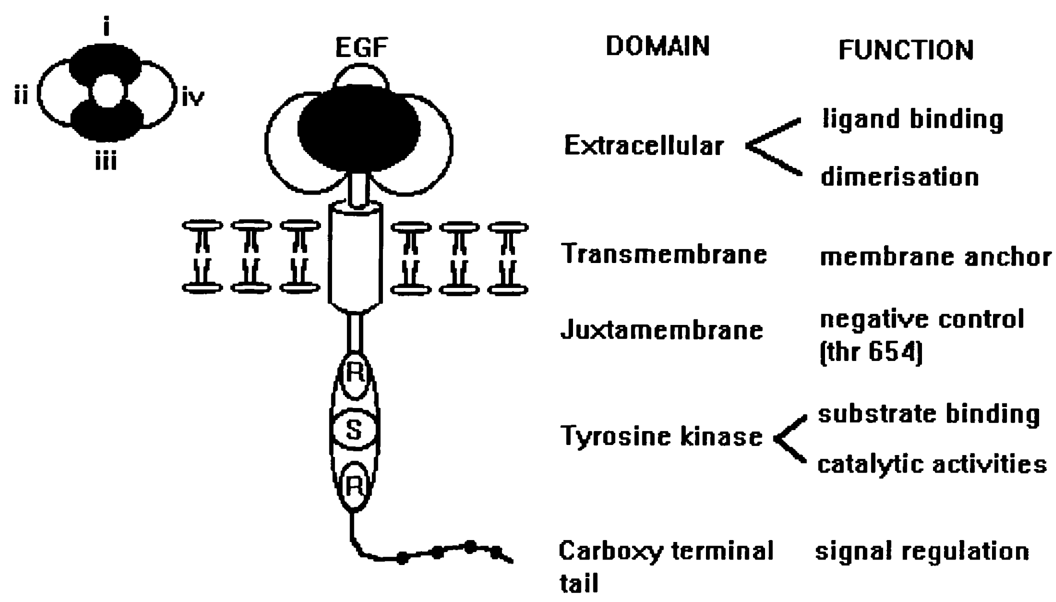
The table shows the amino acid homologies of the four receptors of this family. Domains i and iii represent the sequences between the two cysteine rich regions, ii and iv. TK is the tyrosine kinase domain. Modified from 'Type I growth factor receptors: an overview of recent developments', Mason S and Gullick WJ, The Breast, 4:11-8,1995.

Section ii: Structure of EGF receptor

The EGF receptor is a 170 kiloDalton transmembrane glycoprotein receptor that is expressed on the surface of all cell types, with the exception of differentiated haematopoietic cells. It comprises 1186 amino acids that are encoded by the cellular oncogene c-*erb*B-1, located on human chromosome 7q21. It can conveniently be thought of as having three domains: a 621 amino-acid extracellular domain responsible for ligand recognition; a 23 amino acid hydrophobic transmembrane region; and a 542 amino-acid intracellular region containing the highly conserved tyrosine kinase domain. This topology means that, unlike water soluble allosteric enzymes, the ligand binding and tyrosine kinase domains are separated by the cell membrane. Therefore, receptor activation due to extracellular ligand must be translated, across the membrane barrier, into activation in the intracellular domain.

The extracellular moiety is substantially glycosylated and comprises two pairs of putative domains; globular, or L, and cysteine-rich, or S. It is proposed that these pairs of subunits form a ligand 'pocket'. Ligand binding alters the interaction between these subunits, thus allowing transfer of an allosteric conformational transition to the internal domain (reviewed in Ullrich 1990). A single amino acid helix forms the transmembrane domain. Its main function is to anchor the receptor to the plasma membrane, thereby connecting the extracellular environment with the internal compartments of the cell. The protein kinase domain is located in the intra-cellular portion. A kinase negative mutant of the receptor was unable to propagate intracellular signalling when bound by ligand (Honegger 1987, 1987[1], Chen 1987), inferring that EGF receptor signalling is dependent on tyrosine phosphorylation of cellular substrates. Thus, the tyrosine kinase domain is prerequisite for signal transduction and induction of both early and delayed cellular responses; including mitogenesis and transformation.

Figure 1 2 : Proposed structure-function topology of the EGF receptor.



Subdomains, ii and iv, represent the cysteine rich regions of the extracellular domain. Most of the structural determinants that define EGF binding are proposed to be located in the pocket formed by subdomains i and iii. Far left is a top view of the external domain (not to scale). The symbols, S and R, represent proposed interaction sites for substrates and regulatory factors respectively. Modified from 'Signal transduction by receptors with tyrosine kinase activity' Ullrich A and Schlessinger J, Cell 61:203-12,1990.

Within the intra-cellular portion, flanking the protein kinase domain, are the juxta-membrane region and the carboxy-terminus tail, both of which may influence the receptor's tyrosine kinase activity. The former includes regions, with serine and threonine residues, that can be phosphorylated by intracellular protein kinases, such as mitogen-activated (MAP) kinase and protein kinase C (PKC). Phosphorylation of these sites may regulate EGF receptor activity, internalisation rate and ligand affinity, however, the subtleties of signal regulation, both in terms of phosphorylation sites, and serine/threonine kinases, are still to be determined.

The carboxy-terminus tail is thought to interact with the protein kinase region modulating its capacity to interact with exogenous substrate. This may be effected by competition, for binding of the signal transducing tyrosine kinase region, between intrinsic (carboxy-terminus tail) autophosphorylation sites and exogenous substrate. Such signal modulation has been observed in other tyrosine kinase receptors where carboxy-terminal tail sequences exert a negative control on receptor signalling (Roussel 1987). Adding support to this proposal are studies of mutant receptors with altered autophosphorylation sites, these were mitogenically more responsive to lower doses of EGF when compared to wild type receptors (Honegger 1988[1]). Similarly, other receptor types with carboxy-terminal deletions have increased oncogenic capacity (Khazaie 1988).

Section iii: EGF receptor ligands

There are five cognate ligands to the EGF receptor; EGF, TGF-α, heparin-binding EGF, betacellulin and amphiregulin (Davies 1996). Initially isolated from extracts of mouse submaxillary glands, EGF is a 53 amino acid peptide (Savage 1972). It is characterised by its ability to stimulate precocious eyelid opening and incisor eruption by stimulation of epidermal growth and keratinisation (Carpenter 1979). Subsequently, the human equivalent, urogastrone, was isolated from urine (Gregory 1975).

EGF binds exclusively to EGF receptor and does not directly interact with the other type I tyrosine kinase receptors. It is a potent stimulator of cell proliferation and has effects on the differentiation and function of a variety of cell types (Gregory 1975, Hommel 1991). It has also been shown to reduce gastric acid secretion, to protect against mucosal damage in the gastrointestinal tract, and to have functions in embryo/foetal development (reviewed in Prigent 1992 and in Wiley 1995). Less is known of its role in neoplasia although there are reports of elevated levels of expression in a variety of carcinomas, including gastric and breast (reviewed in Prigent 1992).

Sequence analysis of the EGF molecule has revealed the presence of six cysteine residues which participate in the formation of three disulfide bonds which produce a three-ringed conformation in the peptide molecule (Savage 1973). This three looped structure characterises all members of the EGF receptor ligand family. Besides conserving the cysteine residues that confer this loop structure, all five ligands conform to the motif; $X_nCX_7CX_{4/5}CX_{10}CXCX_5GX_2CX_n$, where X is any non-cysteine residue and n is a variable number (Prigent 1992).

TGFα, a 50 amino-acid peptide that exhibits approximately 40% homology to the EGF molecule, also functions exclusively through the EGF receptor (Todaro 1990). This ligands relationship to the neoplastic process is better documented than that of EGF. An increase in the synthesis and secretion of TGFα occurs in a number of carcinoma cell lines, including human breast lines (Perroteau 1986, Bates 1988), and has been reported in fibroblasts and epithelial cells that have been transformed with a number of different oncogenes (Salomon 1990, Jakowlew 1988, Ciardiello 1990). In up to 70% of breast cancers, wild type TGFα can be localised to the malignant cells (Ciardiello 1989). Further, compared to TGFα levels in benign breast tissues, those in pre-malignant and malignant breast are 2-3 times higher (Parkam 1992, Macais 1989, Lundy 1991, Umekita 1992).

In-vitro, TGFα is a potent mitogen for normal and malignant mammary epithelial cells, and *in-vivo* it stimulates lobulo-alveolar development of the mouse mammary gland (Bates 1990, Vondherhaar 1987). Oestrogen receptor negative cell lines, models for clinically aggressive oestrogen receptor negative breast cancers, tend to express more TGFα than do oestrogen receptor positive breast cell lines (Perroteau 1986, Bates 1988). Futher, in oestrogen receptor positive lines, oestrogen treatment enhances TGFα expression (Bates 1988), an effect that can be blocked by anti-oestrogens (Bates 1988, Murphy 1989). Finally, breast cancer patients treated with the anti-oestrogen Tamoxifen demonstrated a 10 fold reduction of tumour levels of TGFα (Gregory 1989). Summated, these findings implicate TGFα in the progression of malignant breast disease.

Like TGFα, amphiregulin exerts its biological activity through the EGF receptor (Johnson 1993). High levels of this protein have been found in a number of normal human mammary epithelial cell lines (Kenny 1993, Shoyab 1989) where it is thought to stimulate proliferation via an autocrine loop. This hypothesis is leant support by the finding that, EGF-independent, autonomous proliferation of these cell strains is completely blocked by the addition of heparin which binds amphiregulin inhibiting its interaction with EGF receptor (Li 1992, cook 1992).

Additionally, TGFα functions as an autocrine growth factor in c-Ha-*ras* transformed MCF-10A cells, and an antisense oligonucleotide to amphiregulin DNA blocked anchorage dependent and independent growth in these cells (Ciardiello 1990). The implication is that autocrine loops might induce chronic receptor stimulation and that activation of such loops could be important in the progression to malignancy. This concurs with the results of experimental work; elevated levels of both ligand and receptor being associated with cell transformation (Lippman 1986, DiMarco 1989). Furthermore, the majority of breast cancers that produce high levels of TGFα coexpress EGF receptors, suggesting that an autocrine loop may be operative *in-vivo* (Lundy 1991, Umekita 1992).

Autocrine loops may be more prevalent than is presently appreciated since there may be unrecognised EGF receptor ligands. Additionally, ligands may signal by juxtacrine stimulation, a from of cell to cell communication proposed by Massague (1990). The mRNA for EGF encodes a 1217 amino acid precursor molecule which resembles a transmembrane receptor (reviewed in Prigent 1992). In a membrane bound form, this is capable of stimulating EGF receptors on adjacent cells (Dobashi 1991). This scenario is pertinent for the other cognate EGF receptor ligands, all of which are cleaved from larger precursor molecules which resemble cell surface receptors. Consequently, these ligands may mediate cell-cell interactions *in-vivo*, a role which has been confirmed for the membrane bound TGF-α precursor which functions in a juxtacrine fashion during vertebrate development (Paria 1994).

Section iv: Signal transduction

Extracellular ligand binds the monomeric receptor, inducing receptor dimerisation (Schlessinger 1988). This action increases the catalytic activity of the receptor tyrosine kinase (Yarden 1987) and results in the formation, at the cell membrane, of a multi-molecular complex containing cytoplasmic signalling proteins. Signal transduction is achieved by ligand dependent autophosphorylation of, at least, three major sites; tyrosine residue numbers 1068, 1148 and 1173 (Downward 1984[1]). Mutational analysis experiments indicate that phosphorylation of all of these sites is required for substrate access to the receptor's catalytic site (Velu 1989, Helin 1991). Once activated, the kinase phosphorylates a number of intracellular substrates, including, amongst others, phospholipase C-γ, MAP kinase, Raf-1 and GAP (reviewed in Merlino 1990 and in Wiley 1995). Phosphorylation of these effector substrates, together with changes in the intracellular milieu, precipitate nuclear signals activating the transcription of early genes, like *fos* and *jun*, that code for proteins capable of modulating changes in gene expression (Heldin 1987, Nigg 1990). Subversion of these regulatory pathways has been shown to result in malignant transformations (Heldin 1987, Nigg 1990, Fidler 1990).

After ligand binding, receptors on the cell surface are internalised into coated vesicles and rapidly degraded in the lysosomal compartment (Schlessinger 1988). This process of excluding the receptor from the cell surface may provide a mechanism of control over receptor function; truncated receptor, unable to internalise but with normal kinase activity, caused increased transforming activity (Wells 1990). Similarly, a single point mutation, in the transmembrane region of the c-*erb*B-2 receptor, caused cellular transformation, possibly by stabilising receptor dimerisation in the absence of ligand (Weiner 1989).

The above outline of ligand binding receptor producing a given signal, is a gross oversimplification of ligand-induced EGF receptor stimulation. It is clear that the EGF receptor is at the hub of a web of intracellular signals, many of which remain to be elucidated. It is also clear that the receptor signal can be induced in a number of complex manners and that, once generated, signal impinges on a myriad cellular processes. Some of the complexities of EGF receptor signalling are outlined below.

Why does one receptor have five ligands? As a result of variations in their amino-acid content, the different ligands have different isoelectric points. Therefore, the local environment, which is often chemically hostile e.g. gut or infection sites, will determine the cells' ability to respond to a certain ligand (reviewed in Davies 1996). Ligands may also differentially influence receptor signalling via their variable N-terminus. The N-terminus of heparin-binding EGF functions in this manner; binding it to cell surface molecules, thus increasing EGF receptor binding affinity by up to two orders of magnitude (reviewed in Davies 1996). Different ligands may also evoke different biological responses. Amphiregulin, unlike TGF-α and EGF, fails to synergize with TGF-β to promote anchorage independent growth (Shoyab 1989). Similarly, amphiregulin fails to stimulate epidermal to mesenchymal transition in colon cancer cells lines that respond in this manner to EGF (Solic 1995). Also, the different ligands are the products of different cellular processes and ligand stimulation of EGF receptor may reflect only one of the outcomes of these processes.

Untangling the intracellular signalling, and biological outcome of receptor stimulation, is further complicated by the EGF receptor's ability to heterodimerise with other members of the type I tyrosine kinase receptor family. Heterodimerisation was first demonstrated between the EGF receptor and c-*erb*B-2. Kokai and colleagues showed that NIH3T3 cells were not transformed by moderate levels of c-*erb*B-2 unless accompanied by similar levels of EGF receptor expression (Kokai 1989), implying that the receptors were acting synergistically. These receptors were then observed to form heterodimers in a ligand-dependent manner (Spivak-Kroizman 1992, Quain 1992). In a similar fashion, EGF receptor can interact with other type I tyrosine kinase receptors (Soltoff 1994).

Heterodimeric associations may influence downstream signalling in various ways (reviewed in Earp 1995). In comparison to homodimers, heterodimers may:

i. expand available substrate options thus broadening potential signalling pathways,
ii. promote interaction of substrates that would not be juxtaposed by homodimer receptor associations,
iii. alter duration of receptor signalling; altering rates of internalisation, kinase activation, ligand loss etc.,
iv. alter rates of receptor phosphorylation and dephosphorylation.

By any of these mechanisms, individually or in combination, EGF receptor signal transduction may be modified (Wada 1990).

Interpretation of ligand/receptor interaction may be further complicated by events at the receptor level. By a process termed transmodulation, heterologous ligands can phosphorylate threonine residues on the EGF receptor. By this mechanism, platelet-derived growth factor has been shown to influence activity of the EGF receptor (Lin 1986). Similarly receptor inhibition and internalisation have been linked to phosphorylation at threonine 654 (Countaway 1990) and 669 (Heisermann 1990) respectively.

All of the above levels of EGF receptor control are at the extracellular and membrane levels. There is evidence that suggests a further level of control occurs at the cytoplasmic level, via specific cytoplasmic signal transducers, e.g. the nonreceptor protein tyrosine phosphatase SHP-2/Corkscrew (Allard 1996, Perkins 1996) which has been implicated in a number of receptor tyrosine kinase signalling pathways. At present, relatively little is known of this level of signal control but these may further add to the complexities of unravelling EGF receptor function.

Figure 1.3 is a diagrammatic summary of the events following ligand/receptor binding.

Section v: Effect of Signalling

The processes regulated by receptor tyrosine kinases and their ligands are also diverse. They include regulation of cell growth, differentiation, migration, viability and homeostasis. Some receptor tyrosine kinases are cell type specific and are devoted to a single function, e.g. drosophila *Sevenless* which specifies the differentiation of one photo-receptor in the adult eye. Conversely, signalling by others is implicated in a myriad processes. The EGF receptor conforms to the latter category; in drosophila, during just the embryo phase, it is involved in the establishment of ventral cell fates, maintenance of amnioserosa, and ventral neuroectodermal cells, germ band retraction, cell fate specification in the nervous system and production of cuticle (reviewed in Perrimon 1997).

How does a single tyrosine kinase receptor regulate such a diversity of processes? It has been suggested that multiple ligands might provide modulation of EGF receptor activity (Shoyab 1989, Solic 1995). Diversity in the expression of these ligands as well as a control over their activity has increased the repertoire of receptor functions (reviewed in Perrimon 1997). Alternative hypotheses are: that EGF receptor function is the result of a balance of negative and positive feedback loops which will vary in their emphasis dependent on the particular cells in question, this has been demonstrated by work on cell fate in drosophila ventral ectoderm (Golembo 1996); or that the EGF receptor may function as an on-off switch with the result of its activation determined by the cells on which it acts, as seen in gene expression patterns in the ventral ectoderm of drosophila, (Golembo 1996).

The sections above focus on the molecular level of EGF receptor function. Much remains to be learnt of the functional significance of EGF receptor stimulation before its clinical importance will be appreciated. To date, most attention has

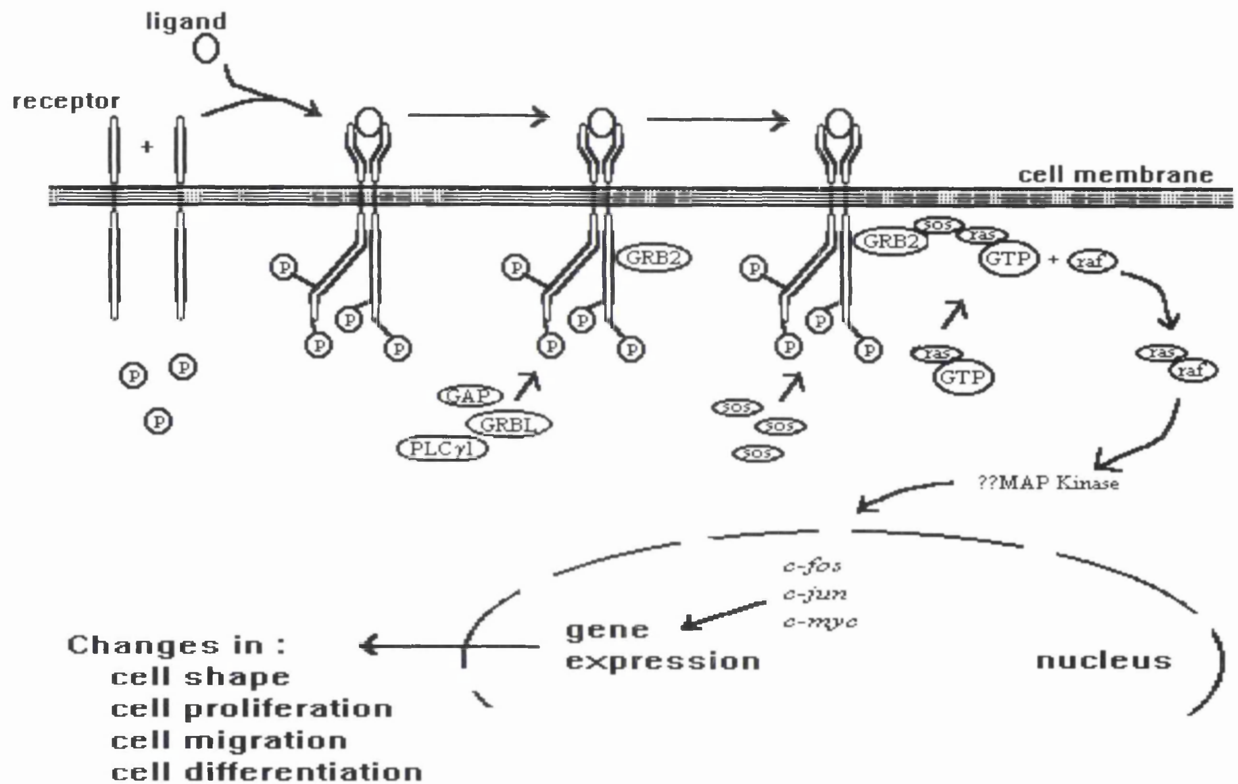


Figure 1.3 : Summary of the EGF receptor signal transduction pathway.

When the ligand binds to the EGF receptor it dimerises and undergoes autophosphorylation. This creates high affinity binding sites on the cytosolic domain of the EGF receptor for effector proteins possessing a 100 amino acid motif known as the SH2 (src homology 2) domain. These include GAP, which stimulates *ras*p21; PLC-γ, which enzymatically cleaves phosphotidylinositol-4,5-bisphosphanate into the two secondary messengers inositol-1,4,5-trisphosphate and diacyl-glycerol; and GRB2, which bridges the association of the EGF receptor with a guanine-nucleotide releasing factor SOS. Recruitment of SOS to the plasma membrane by GRB2 approximates SOS to its substrate, c-*ras*p21, which interacts with yet another proto-oncogene, *raf*, to transduce EGF receptor generated signals, possibly via MAP kinase. This cascade of serial interactions eventually effects the expression and activity of nuclear transcription factors including c-*fos*, c-*jun*, c-*myc* , to result in changes in cell proliferation, cell migration and/or cell differentiation. Modified from 'EGF receptor function in early mammalian development' Bioessays 17: 840, 1995.

focused on the promotion of mitogenesis since there is good evidence from *in-vitro* studies that EGF receptor stimulation effects this response (Stoscheck 1986, Hamburger 1981, Singletary 1987). Further, the growth of A431 cells transplanted to athymic mice correlates directly to the degree of gene amplification and the concentration of EGF receptors (Santon 1986) and the growth of human gastric carcinoma xenografts, speeded by the addition of EGF, can be suppressed by sialoadenectomy (Okuda 1994). The implication is that EGF stimulation of EGF receptor results in proliferation. However this may be simplistic. Chorioepithelioma xenografts in nude mice treated with EGF demonstrate a biphasic response; low EGF concentrations promoting proliferation whilst this response is inhibited by higher concentrations (Myachi 1990). Also, addition of EGF to subcutaneous tissues adjacent a breast cancer xenograft inhibits its growth (Murayama 1990) and both EGF and TGFα can inhibit proliferation of some cell lines depending on the presence of other growth factors and the levels of EGF receptor (Shoyab 1988). Therefore, the EGF/EGF receptor axis mediates effects more complex than a simple promotion of mitogenesis.

Examination of the events following receptor stimulation does not clarify the situation. Nerve growth factor (NGF), also a tyrosine kinase growth factor receptor implicated in oncogenesis, stimulates differentiation but inhibits proliferation of the rat phaeochromocytoma clone, PC12 (Tischler 1975). The same clone is stimulated to proliferate by EGF (Huff 1981). However, there are no discernible differences in the early response genes activated by stimulation of both receptors (Greenberg 1985, Bartel 1989) and a variety of diverse reactions appear similarly induced; membrane ruffling (Seeley 1984), Na^+/K^+ transport (Boonstra 1983), cell adhesion and deoxy-glucose uptake (Huff 1981). Even the pattern of tyrosine phosphorylation of cellular proteins is very much alike (Maher 1988). Put simply, NGF and EGF trigger the same set of early responses, none of which are wholly specific for EGF or NGF. Therefore, the steps in signalling for neurotrophic effects appear to involve the same molecules used for the transduction of mitogenic signals. How then is the specificity of growth factor/receptor signalling achieved? It is possible that it is a consequence of parallel discriminatory events that have not been elucidated or events further downstream that are more important in determining the divergence of the signalling pathways. Equally, EGF receptor function may be determined, or influenced, by the particular cellular context. Amongst other functions are an immunosuppressive effect, reducing host resistance and accelerating cancer growth and metastasis (Koch 1984), or increased tumour cell invasiveness, a possibility that is considered further in Chapter 4.

Since its discovery in 1984, a great deal has been learnt of the EGF receptor and its signalling, however much remains to be elucidated. From the account above, it is clear that we have an incomplete comprehension of the ligand-receptor interaction event, the resultant signal, its modulation and also its functional effect. Overall, our knowledge of the functional significance of expression levels of receptor, in malignant and indeed normal, tissues remains crude.

Chapter 2:
EGF receptor expression in breast cancer

Section i: Early studies of EGF receptor in breast cancer

Beatson, as early as 1896, had hinted at the hormonal dependence of some breast cancers (Beatson 1896). Subsequently, oestrogen receptor expression was observed to influence breast tumour progression; oestrogen receptor positive tumours having improved prognosis over oestrogen receptor negative tumours. Oestrogen receptor status also influenced the site at which metastases tended to develop; oestrogen receptor negative tumours metastasised to liver and brain whereas those of oestrogen receptor positive tumours showed a propensity for soft tissue and bone (Stewart 1981). With these findings, there was growing evidence that tumour cell biology was, at least partly, regulated through receptor signalling.

Cooke (1982) used thymidine labelling to demonstrate that oestrogen receptor negative tumour cells proliferated more rapidly than their receptor positive counterparts. Yet EGF was a more potent mitogen than oestrogen in breast cancer cell lines (Osborne 1980). Further, during breast development and maturation, EGF was present, at high levels, in both tissue and plasma, and human milk was also rich in this moiety (Carpenter 1980). EGF receptor, therefore, has a role in normal breast development, but, with its substantial homology to the v-*erb*B oncogene product, was it also functioning in breast tumour biology?

The first reports of EGF receptor expression in human breast cancers were in 1985 (Sainsbury 1985, Sainsbury 1985[1]). By demonstrating a higher frequency of receptor posisitvity in metastatic lymph node specimens than in primary breast tumours, Sainsbury's group suggested that its expression correlated with metastatic potential. They also reported an inverse relationship between oestrogen receptor and EGF receptor expression and proposed that the growth of a proportion of oestrogen receptor negative tumours might be regulated via the EGF receptor. This publication concluded that it might be possible to regulate the growth of some human breast cancers by interfering with EGF receptor function.

In the same year, Sainsbury reported that there was a strong correlation between EGF receptor expression and Bloom and Richardson score; $p<0.01$ in a series of 108 ductal carcinomas. This led to the hypothesis that measurement of EGF receptor might provide a useful prognostic indicator (Sainsbury 1985[1]). In 1987 the same group published the results of follow-up on a prospective series of 135 patients (Sainsbury 1987). They observed that relapse free survival and overall survival were significantly worse in those patients with EGF receptor positive carcinomas, as opposed to those that were receptor negative. The converse was shown for oestrogen receptor status. Further, a subgroup of patients with oestrogen receptor negative/EGF receptor positive tumours had the poorest prognosis. Multivariate analysis showed that, in lymph node negative tumours, EGF receptor was the single most important predictor for relapse-free and overall survival, and that in node positive disease it was the second most important, after nodal involvement. They concluded that EGF receptor expression was the most important variable for predicting relapse-free and overall survival (Sainsbury 1987).

Section ii: EGF receptor; relationship with other prognostic factors

Since these early studies, EGF receptor expression, and function, in breast carcinoma have been studied extensively. Despite this, there is still no clear consensus on the clinical relationships and prognostic value of the measurement of this receptor in breast cancer. Its clinical significance in this pathology is most thoroughly reviewed by Klijn *et al* (1992). This author trawled the literature for data on clinical parameters and receptor expression, the resultant publication reports the study of 5232 patients from studies by 40 different research groups. Such meta-analysis can be difficult to interpret (Ioannidis 1998), but by limiting the analysis to the largest and most recent studies and by combining only the results of comparable studies, Klijn has provided a useful overview.

a) Relationship to oestrogen receptor

Despite a wide variation in the reported levels of both oestrogen receptor, 34-82%, and EGF receptor, 14-91%, at least twenty-eight different groups (reviewed in Klijn 1992) have reported an inverse relationship between these receptors. Similarly, twelve of nineteen groups reported an inverse relationship between progesterone and EGF receptors. Subsequent to Klijn's review, Koenders *et al* (1991) published on steroid hormone and EGF receptor levels in 531 breast cancers, easily the largest single series. They observed that not only was EGF receptor positivity more frequent in oestrogen receptor negative tumours ($p<0.0001$) but that levels of EGF receptor were also significantly higher in this group ($p<0.0001$). In the same paper, a similar relationship was reported between EGF and progesterone receptors. Thus there is a relative concensus regarding the relationship between expression of EGF receptor and steroid hormone receptors.

b) Relationship to tumour size

With regard to tumour size, fourteen groups failed to identify any association with receptor density. However, two groups, including Sainsbury's, found a direct correlation between receptor expression and tumour diameter. Contradicting this are the reports of three groups, including Klijn's (Foekens 1989), each of which reported an inverse relationship between these parameters.

c) Relationship to lymph node metastasis

Following breast cancer surgery, the presence of lymph node metastasis has traditionally provided the best indication of prognosis (Valagussa 1978, Fisher 1983). For this reason considerable interest has focused on the relationship between lymph node metastases and EGF receptor levels, however, this is one of the areas where there is greatest contradiction in the literature. In his 1987 publication, Sainsbury, observed that the frequency of EGF receptor positivity was higher in node-positive disease than in node negative cancers. It is however worth noting that, in this series, only 52.6% of patients had lymph nodes biopsied; the study protocol required that all patients undergoing local excision have nodes biopsied, but if undergoing mastectomy, only those patients with palpable nodes had these biopsied. In accord with this report are the findings of Battaglia (1988), who reported 77.7% EGF receptor positivity in node positive carcinoma, as opposed to only 25.7% in node negative disease. Bolufer (1990) demonstrated a similar relationship but only in oestrogen receptor positive carcinomas. Others have suggested a linear relationship between levels of receptor, or gene transcript, and numbers of involved nodes, in all tumours (Hainsworth 1991) and in non-inflammatory breast cancers (Guerin 1989). Contradicting these observations was the report of Grimaux (1989), finding that EGF receptor levels were elevated in those tumours with less than four involved nodes. Perhaps more significantly, a further fifteen groups failed to demonstrate any correlation between, either nodal involvement, or numbers of involved nodes, and receptor levels.

As is indicated above, Sainsbury's initial paper reported higher levels of receptor expression in lymph node metastases than in primary cancers, an observation used to promote the hypothesis that receptor expression correlated with metastatic potential. This finding, of higher receptor expression in metastases than primary disease, is less contentious than the relationship between receptor and nodal status. It enjoys the support of several other publications (Battaglia 1985, Macais 1986) but once more there is controversy; Grimaux *et al* (1989), studying a series of 55 breast cancers, could not confirm this relationship.

d) Relationship to histological type

The vast majority of invasive breast cancers are histologically classified as ductal carcinomas, the next largest subgrouping is lobular carcinoma, accounting for approximately 12% of all breast cancers (Wright 1986). The remaining sub-types of breast cancer make up only a small percentage of total cases. A number of studies have reported higher EGF receptor expression in ductal carcinomas than in lobular types but equally others have failed to confirm this association (reviewed in Klijn 1992). Better differentiated breast tumours, including tubular and mucoid cancers, have been reported as expressing EGF receptor, but usually at lower levels than ductal carcinomas (Skoog 1986, Sainsbury 1988). An exception, is the well differentiated breast carcinoma of medullary type. In Klijn's review, only eight of 831 histologically typed carcinomas, were classified as medullary but seven were considered EGF receptor positive. However, using an immunohistochemical estimation of EGF receptor, Moller (1989) found receptor positivity in only one of seven medullary carcinomas.

The 831 patients, reviewed by Klijn, encompass the results of six separate studies. In summary; 658 tumours were classified as ductal and the proportion of EGF receptor positive tumours ranged from 23% to 58% (overall 35%), 85 were classified as invasive lobular carcinoma, with receptor positivity in 0% to 57% (overall 28%), with 43 carcinomas classified as 'other', EGF receptor positivity ranging from 13% to 67% (overall 37%). Thus, there was no clear relationship between EGF receptor expression and the histological type of breast cancer.

e) Relationship to hitological grade

Histologically, invasive ductal carcinoma varies considerably. In 1957, Bloom and Richardson proposed a histological grading system (Bloom 1957), and subsequently showed this grade to be of prognostic significance (Bloom 1971). A modification of this system, remains the basis of breast tumour grading, as advocated by the World Health Organisation (WHO 1968). Tumours are graded, from one to three, depending on three features of the carcinoma cells; tubular formation, mitotic index and nuclear pleomorphism. Higher grade is associated with poorer prognosis; 81%, 54% and 34% five year survival for grades one, two and three respectively (Bloom 1971). Of the 40 reports reviewed by Klijn (1992), 19 included studies of tumour grade. Ten of these reports described a statistically significant association between poorer grade and EGF receptor expression, however, eight failed to determine such an association. In the remaining report, this relationship was observed only in those tumours that were oestrogen receptor negative (Bolufer 1990).

f) Relationship to other parameters

Klijn also reviewed EGF receptor expression in terms of other, less frequently used, prognostic indicators. These included flow-cytometric assessment of tumour ploidy and parameters of cellular proliferation. Seven studies addressed the association between receptor expression and ploidy. The numbers of tumours in these studies was relatively low, and maybe not surprisingly, given the variation of reported associations between receptor expression levels and the more commonly measured prognostic indicators, there was some discord between these studies. Whilst all seven suggested that EGF receptor levels were higher in aneuploid tumours, in only one study was this statistically significant (Walker 1986). In total 367 tumours were analysed, overall 35% of aneuploid tumours, and only 15% of diploid tumours, were reported as receptor positive. Klijn (1992) used this combined analysis to suggest that EGF receptor positivity occurred more frequently in aneuploid tumours, and that individual investigators had failed to find this association statistically significant due to the small size of their respective studies. This is a problem that afflicts many of the publications on EGF receptor.

A number of parameters reflecting cellular proliferation rates have been described, these include thymidine labelling index, S-phase fraction and Ki-67. Klijn reviewed nine reports of indices of proliferation. Three studies identified statistically significant associations with EGF receptor expression. In one, Ki-67 staining was observed at higher levels in EGF receptor positive tumours (Toi 1990). However, this publication reported on just 27 tumours, and three larger studies have failed to confirm this finding. In another, S-phase fraction correlated with receptor expression (Walker 1986) but two subsequent studies failed to confirm this observation. The last of the statistically significant reports, showed a correlation between numbers of mitoses, counted by light microscopy, and EGF binding (Spitzer 1987), but, in a report the following year, this author indicated that this association was chiefly in node positive tumours (Spitzer 1988). Reports, subsequent to those in Klijn, have left the relationship, between EGF receptor expression and indices of proliferation, no less confused. Ki-67 staining has been reported to correlate with EGF receptor expression by some groups (Nicholson 1993, Gasparini 1992, Charpin 1993), but not by others (Gasparini 1991).

S-phase fraction is subject to the same incongruities; new studies (Gasparini 1991, Minckwitz 1993) failing to confirm that of Walker (1986).

In summary, of the universally applied prognostic criteria only the relationship between EGF receptor and steroid hormone receptor status seems to be clear. There is no consensus regarding EGF receptor expression and either tumour size or nodal status, but it would appear that receptor levels are higher in metastatic than primary disease. Tumour grade is increasingly frequently reported in routine pathology, and there appears to be an association between poorer grade and receptor expression. Of the less frequently measured prognostic indicators the literature abounds with conflicting reports, but this may, in part, reflect the small size of many of these studies and variation in the means of determining EGF receptor positivity. Overall, for most routinely assessed prognostic indicators, there is some inter-study concordance, but individual publications are frequently at odds.

Section iii: EGF receptor; relationship with outcome in breast cancer

a) Critique of published reviews

The relationship between EGF receptor and outcome is also controversial. Klijn's review of outcome, both overall survival and disease free survival, encompassed the results of eleven studies from nine research groups, including that of Sainsbury (1987). Klijn reports a wide range of both the duration of follow up and the published levels of EGF receptor positivity; median of 12 to 66 months and 14% to 55% of tumours respectively. This stated, using univariate analysis, five of these groups found a statistically significant relationship between receptor positivity and relapse free survival or overall survival (Rios 1988, Nicholson 1990, Costa 1988, Grimaux 1989, Lewis 1990). Macais (1987) had previously reported a study of 72 patients but had failed to observe a significant association between receptor levels and outcome, and although this groups' second report (Rios 1988) identified a relationship between receptor positivity and outcome it could be criticised for its short duration of follow-up (maximum of 30 months).

A similar complaint may be levelled at the studies of Costa *et al* (1988) and Lewis *et al* (1990), with median follow-up of 12 and 18 months respectively.

Two of these five groups were able to confirm statistical significance of the relationship between receptor expression and outcome using a multivariate analysis (Sainsbury 1987, Lewis 1990). However, the only study to identify a statistically significant relationship with overall survival using a multivariate analysis was that of Sainsbury (1987). This publication states that EGF receptor expression was the single most important predictor of disease free, and overall, survival. As indicated above, this may, in part, be due to the study protocol which did not require that nodes be biopsied in all patients. This complaint might also be levelled against a later report from the same group (Nicholson 1990); in this study lymph nodes were biopsied only if palpable (56% of cases) and for the purposes of the multivariate analysis impalpable nodes were assumed to be negative. It is not stated in Sainsbury's 1987 publication if tumours with clinically impalpable nodes were considered node negative, but, if so, this could bias the multivariate analysis, particularly given the difficulties of assessing axillary lymph node involvement clinically (Smart 1978). Harris (1989) hinted at the frailty of this approach but defended it on the premise that not all surgeons performed routine axillary lymph node biopsy and therefore the prognostic features which could be determined from the primary should be considered paramount. This publication (Harris 1989), of a series of 203 ductal carcinomas, reported a multivariate analysis including tumour size, grade, EGF receptor and oestrogen receptor status, only EGF receptor and size proved of predictive value. However, lymph node biopsy was not performed in 97 of the 203 patients. Excluding this cohort, node status was the only predictor of overall survival and disease free survival, size predicted disease free survival and EGF receptor status only showed a trend toward predicting disease free survival. This second analysis is also difficult to interpret. It cannot be considered to reflect the results that might be obtained for all cancers since it includes only those patients undergoing lumpectomy or those with palpable nodes proceeding to mastectomy. The criteria used to determine the surgical procedure, mastectomy of lumpectomy, are not made clear, and the analysis was performed on a cohort likely to have a higher incidence of lymph node metastasis than the study series as a whole.

Subsequent to Klijn's review, Fox *et al* (1994), reported on a series of 370 carcinomas, observing that, in multivariate analysis, lymph node status was the only independent prognostic indicator of overall survival. Lymph node status and oestrogen receptor status predicted for disease free survival and EGF receptor was an independent prognostic indicator only for disease free survival in node negative disease. In this study lymph nodes were biopsied in all cases. Gasparini (1994) reported a series of 165 cases, all with lymph node status available, EGF receptor positivity was a significant predictor of disease free survival in node positive and node negative disease but failed to predict death.

In their 1994 review, Fox *et al* (1994) collated the results of 16 studies of EGF receptor expression and outcome, both disease free survival and overall survival. These included reports from the groups reviewed by Klijn (1992), in total, results from 3009 patients were reviewed (compared with 1653 in Klijn's 1992 paper). Of these 16 studies, in 10, using univariate analysis, there was a statistically significant association between receptor positivity and reduced disease free survival or overall survival. Multivariate analysis was rarely performed; five of nine studies employing this technique reported reduced relapse free survival in EGF receptor positive patients, but one group later reported (Gasparini 1994) that with longer follow-up, five years as opposed to three, the significance of EGF receptor expression was lost. In only one of five studies was there a statistically significant relationship between receptor levels and overall survival (Grimaux 1989). Fox hypothesised that, in many studies, lack of an adequate duration of follow-up might be responsible for this observation. His argument is supported by the observation that receptor positivity is more frequently reported to be associated with recurrence than overall survival, but must be tempered by the observation that the discriminative prognostic power of the EGF receptor may reduce with longer follow-up. This observation has been reported for oestrogen receptor (Kinsel 1989), and can also be extrapolated from the published results of Nicholson (1991) where the survival curves approximate with longer follow-up.

b) Analysis of patient subgroups

Many researchers have endeavoured to identify patient subgroups in which EGF receptor expression correlates with outcome. This is most usually addressed by analysing EGF receptor expression in subgroups determined by oestrogen receptor status or lymph node status. The results of these analyses are variable. Some publications report a prognostic significance of EGF receptor expression in oestrogen receptor positive tumours (Nicholson 1990, Rios 1988) others in oestrogen receptor negative tumours (Sainsbury 1987, Lewis 1990, Nicholson 1990), yet others fail to observe a statistically significant relationship in either subgroup (Grimaux 1989, Foekens 1989). For lymph node positive and negative subgroups there are similar incongruities. Some groups found a statistically significant relationship between outcome and receptor positivity in node negative carcinomas (Sainsbury 1987, Nicholson 1990, Spyratos 1990, Fox 1994) others failed to confirm these findings (Macais 1987, Foekens 1989). The contradictory nature of these reports is apparent, not only between studies, but within studies. Fox's study (1994) of 370 patients, reported a statistically significant association between EGF receptor and oestrogen receptor in node-positive tumours, in terms of relapse free survival but not overall survival. Conversely, in node-negative tumours this association was significant for overall survival but not for relapse free survival.

EGF receptor expression has also been analysed in conjunction with less established prognostic indicators. For example, Gasparini *et al* (1994) found that the prognostic significance of EGF receptor was enhanced when combined with measurement of S-phase fraction. In tumours with low S-phase fraction EGF receptor was not useful for predicting relapse, but in those tumours with higher S-phase fraction recurrence was predicted by higher receptor levels. However, they concluded that this finding needed confirmation in a larger, and ideally, prospective series.

One dilemma in the management of breast cancer patients is deciding who should proceed to systemic adjuvant therapy. Those with more advanced disease clearly require further treatment, but identifying those with earlier disease who would benefit from additional systemic treatment remains problematic. This has prompted analysis of this cohort in subgroups defined by perceived risk of relapse. As noted above, in node negative, early disease, Nicholson *et al* (1991) reported that EGF receptor expression identified a cohort of patients who did poorly and might, therefore, have benefited from adjuvant therapy. Two hundred and
thirty-one patients were followed-up for a median of 45 months, in those with node negative disease EGF receptor expression was superior to oestrogen receptor in predicting recurrence and survival. Multivariate analysis of EGF and oestrogen receptor, tumour size and grade, revealed only EGF receptor to be a significant predictor of disease free survival and overall survival in this subgroup. However, Bolla *et al* (1994) published a series of 229 T1/T2, N0/N1 breast cancers with a median follow-up of 34 months. In this study, no EGF receptor cut-off value proved significant in univariate analysis. Multivariate analysis revealed only tumour size, nodal status and grade to prove significant predictors

of disease free survival. Included in this analysis were measurement of oestrogen receptor and progesterone receptor, EGF receptor expression proved the least significant prognostic variable.

c) Response to endocrine therapy

Although patients with oestrogen receptor positive tumours live longer than their oestrogen receptor negative counterparts, the role of oestrogen receptor in predicting disease free survival and death is controversial (Howell 1984). This is partly due to the fact that oestrogen receptor positive tumours tend to respond much better to endocrine therapies; 65% responding as opposed to 15% of oestrogen receptor negative tumours (DeSombre 1980). If oestrogen receptor positive tumours fail to respond to endocrine therapy then the outcome is similar to that of oestrogen receptor negative tumours (Howell 1984). The same problem may apply to interpretation of EGF receptor expression. Wright *et al* (1992) published a study showing a lack of response to Tamoxifen therapy in those breast cancer recurrences expressing EGF receptor. Nicholson (1994) found this loss of endocrine sensitivity in primary disease and reported that it was at least partly quantitative; tumours with higher EGF receptor density responding less than those with some receptor expression, these in turn showing a propensity to relapse more quickly than receptor negative tumours. It is tempting to attribute these results to the inverse relationship between EGF and oestrogen receptors. Yet, although Nicholson's study reported this well established inverse relationship, it also noted that within oestrogen receptor positive and negative cohorts EGF receptor expression maintained its predictive effect. They also found oestrogen receptor negative/EGF receptor positive cells that did respond to endocrine therapy, postulating that they did so through a paracrine effect, or through reversal in phenotype, as is seen with ZR75-9al breast cancer cells on treatment with Tamoxifen (Long 1992). It is worth recalling that normal breast cells, whilst hormonally sensitive, are often oestrogen receptor negative (Walker 1991, Walker 1992), and it may be that in some circumstances endocrine response may be ambivalent with respect to receptor expression. In the same study endocrine response was better in tumours with low rates of cell proliferation, as measured by Ki-67 staining, and the authors concluded that there were a multiplicity of factors influencing a tumours response to endocrine therapy.

The results of EGF receptor measurement in the primary breast tumours of more than 10 000 patients have been published (Harris 1994). Still there is no consensus over the role of EGF receptor in prognosis. Our poor understanding of the relationship between this receptor and both other prognostic factors and outcome is, at least partly, due to the small study size in many of the published series; of the forty studies reviewed in Klijn (1992) only three included more than 200 patients. However, more pertinent may be vagaries in study design, and in particular lack of standardised criterion for determining receptor positivity (Koenders 1992, Klijn 1992, Fox 1994).

Chapter 3:
Measuring EGF receptor expression

Section i: Review of EGF receptor measurement in breast cancer

Much of the discrepancy in the relationships between EGF receptor expression and various prognostic indicators and also outcome may relate to the techniques used to measure this receptor.

In the vast majority of studies, EGF receptor has been measured utilising ligand binding or immunohistochemical methods; 23 and 11 reports respectively in the review of 40 study groups by Klijn (1992) and all but one of the studies, reporting on follow-up, reviewed by Fox (1994). Other methods have been applied to receptor measurement (Klijn 1992). These include; autoradiography (Spitzer 1988, Reubi 1989), immunoenzymatic assays (Grimaux 1990), measurement of EGF receptor transcripts (Guerin 1989, Coombes 1990), but the mean levels of receptor positivity have varied little from those reported for ligand binding or immunohistochemical methods (Klijn 1992). The only method that produced significantly different levels of receptor positivity, 91%, was that measuring EGF receptor phosphotyrosine kinase activity (Baugnet-Mahieu 1990).

Although more established than the other methods, both ligand binding and immunohistochemical techniques may be criticised. Ligand methods provide objective measurement of receptor but are performed on membrane preparations that will include non-tumour components - a deficiency that applies to all methods performed on preparations of tumour samples. Conversely, immunohistochemistry allows specific assessment of tumour cells but is not truly quantitative. Toi *et al* (1994) studied a series of breast cancers using both methods and compared the results. Univariate analysis showed EGF receptor positivity was a significant predictor of relapse free survival using either method. The ligand methodology produced a greater discriminative effect, $p < 0.01$ compared to $p < 0.05$. It also allowed separation of the EGF receptor positive tumours into statistically distinct, high and low, risk groups. This was not possible with the immunohistochemical protocol. Despite this, the authors concluded that the methods were complementary and that neither was superior.

Review of the literature reveals no real differences in the rates of receptor positivity produced by radio-ligand studies and immunohistochemical studies, but between individual studies, using either technique, there is considerable variation. Ligand based studies show EGF receptor expression in a mean of 49% of cases with an inter-study range of 16-91% (Klijn 1992). A review of immunohistochemical studies shows that about 40% of breast tumours express EGF receptor but here also is a wide inter-study range of 14-65% (Klijn 1992). For both of these techniques, this level of disparity is likely to be due largely to methodological variation.

Section ii: Measurement protocols lack standardisation

Lack of protocol standardisation, and its potential influence on receptor studies, has not gone unnoticed. In fact, a number of investigators have made pleas for standardised assay procedures (Koenders 1992, Klijn 1994, Fox 1994). Whilst it has also been suggested that a lack of standardisation makes comparison of literature data 'precarious' (Koenders 1992).

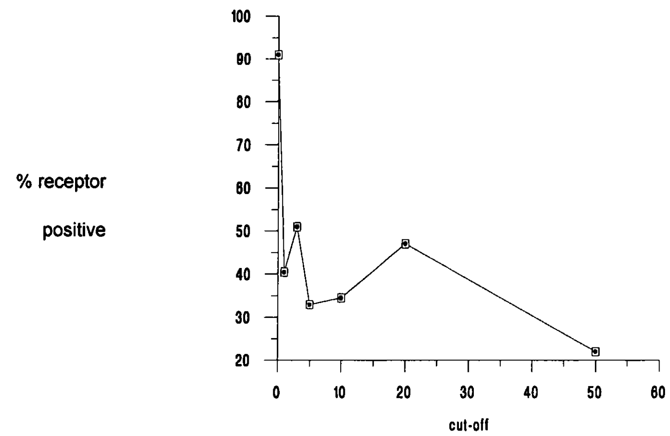
Ligand studies predominate and the objective nature of receptor measurement by this technique has allowed the intricacies of protocol variation to be explored.

a) Defining receptor positivity

Receptor expression is normally reported as positive or negative. The arbitrary nature of this categorisation is clearly demonstrated, although not specifically reported on, by Fox (1994); the 12 ligand binding based studies he reviewed have a cut-off for receptor positivity that varies from any measurable receptor to 50fmol/mg membrane protein (see Figure 3.1).

It might be expected that there would be an inverse relationship between the level taken to represent receptor positivity and the percentage of tumours in the series that proved positive. If the sensitivity of receptor measurement in each study was similar then a plot of these parameters should produce a smooth curve. That it does not (Figure 3.1) indicates that the results of the different studies may not be directly comparable. Adding weight to this argument, Koenders *et al* (1991) reported that, within individual studies, the wide variation in EGF receptor expression rates was not reflected by a similar variation of oestrogen receptor positivity rates. This, in the light of a well documented inverse relationship between these two receptors (see Chapter 2), suggests that methodological differences may be responsible.

Figure 3.1: Relationship between the 'cut-off' for receptor positivity and the percentage of tumours positive for EGF receptor.



The figure shows the results for 12 studies utilising ligand binding techniques (Fox 1994). On the x-axis is plotted the cut-off value (fmol/mg membrane protein) above which the tumours were considered EGF receptor positive. On the y-axis is the percentage of EGF receptor positive tumours in the series. Where more than one study used the same cut-off value the mean percentage of receptor positivity is shown.

b) Assay sensitivity

The lack of an accepted level for receptor positivity assumes greater significance when the sensitivity of the technique is considered. Koenders *et al* (1991) applied a ligand binding method to a series of 531 tumours. In doing so, they identified a direct correlation between receptor positivity and cell membrane protein levels. This relationship was strongest at the lowest membrane protein levels and was interpreted as indicating that the lower limit of the assay sensitivity had been exceeded. Below a cell membrane protein concentration of 0.2mg/ml (equating to a wet weight of biopsy sample of 300mg), the rate of false-negative assays was higher. Applying this limit excluded 27% of the 531 tumour specimens from analysis, and on this basis, they reported a 57% receptor positivity. This is one of the highest rates reported. Clearly, by incorrectly classifying EGF receptor positive cancers as negative, other investigators may have obscured the association between receptor, other pathological variables and outcome.

c) Variable assay design

There are a myriad of variations in EGF receptor measurement protocols. All of the 18 ligand based reports reviewed by Leake *et al* (1993) differed in methodology.

Variation was cited in the:

- method of preparation of the membrane component,
- method of radioiodination of the ligand,
- incubation conditions during the assay,
- ligand concentrations used,
- use of single point or multiple point assays,
- method of separating bound and unbound ligand.

Concerns over the effects of this variability have led to increased efforts to establish standardised protocols. The EORTC Receptor Study Group have declared the hydroxyapatite method as that of choice for separating bound from free ligand (Benraad 1990). Koenders *et al* (1991) have advocated that ligand assays be applied only to membrane preparations containing more than 0.2mg/ml membrane protein, thereby reducing the incidence of false negatives. Foekens (1991) also pointed out that using a multi-point assay technique, as opposed to single point assays, allowed extrapolation of the binding data to the abscissa of the Scatchard plot, producing more accurate assessment of receptor levels. A multi-point assay is now the preferred technique of the EORTC receptor group (Leake 1993). These efforts have culminated in the Commission for European Communities, directorate-general Science, Research and Development, definitive EGF receptor measurement protocol (Leake 1993).

The subjective nature of immunohistochemistry makes the effects of methodological variation more difficult to ascertain but it is likely to be no less important than for ligand binding. The deficiencies of ligand protocols, in terms of defining receptor positivity, assay sensitivity and assay variation, pertain equally to immunohistochemical studies. For instance, if immunohistochemical staining is to be categorised as present, or absent, there is reasonable observer accord, but this concordance disappears if staining is to be graded (van Diest 1996). Similarly, primary antibodies are variable and the signal they produce can be amplified by a multitude of methods. Also, many studies have been performed on formalin-fixed, paraffin embedded tissues. This type of assay suffers from a number of limitations. Firstly, routinely processed material may be fixed for times ranging from a few hours to over a weekend which is likely to result in variable antigenicity. Furthermore, tissue in the centre of a large block will have a lesser degree of fixation compared to tissue at the periphery and this may explain the variation in staining commonly seen across a section. Our own group has reported on this problem in relation to the c-*erb*B-2 encoded receptor (Reeves 1996).

Application of a standardised ligand binding protocol will hopefully allow future studies to clarify some of the confusions surrounding our understanding of this receptor. However, standardised protocols, for ligand and immunohistochemical studies, will not solve all the problems.

Section iii: Conventional measurement techniques are flawed

Standardised ligand binding protocols will not obviate the inherent flaw of this technique. That is, that ligand studies are performed on membrane preparations that are derived from tumour biopsies which will include, besides malignant cells, non-tumour elements, including normal breast, in-situ disease, connective tissue and lymphoid cells. This may not represent a significant problem for the study of tumour types with high EGF receptor levels, e.g. squamous carcinoma of the head and neck (Stanton 1994), but for breast cancers where EGF receptor expression may be closer to, or less than, normal breast tissue (Dittadi 1993), these contaminants may be important. Conventional immunohistochemical methods also fail to escape flaw. The subjective nature of scoring immunohistochemical sections results in a high degree of inter-observer variation and this detracts from the results of this technique (Sallinen 1994, van Diest 1996).

A further problem, for both methods, is the categorical, positive or negative, fashion of reporting receptor status. In cancers, the normal distribution of receptor expression may well be distorted, but, with the caveat of the 3% of tumours with amplification of the EGF receptor coding gene, a simple dichotomy to positive and negative will be arbitrary and is likely to vary depending on the measurement protocol. That a simple dichotomy is almost universally applied probably indicates poor sensitivity, or paucity of accuracy, of receptor quantification using conventional measurement methods. This categorisation simplifies statistical analysis but dilutes the statistical power of a continuum of data. A more accurate measurement method would allow accurate categorisation of cancers or could allow analysis of receptor expression as a continuous variable.

Section iv: Introducing radioiimunohistochemistry

Clearly, the problems associated with the EGF receptor assays may compromise the accuracy of the data obtained from these studies, and therefore, our understanding of the precise relationship between receptor expression, patho-clinical variables and the biology of breast cancer. We have therefore developed a quantitative radioimmunohistochemical method for the measurement of EGF receptor in frozen tissue sections. This assay uses radio iodinated anti-receptor monoclonal antibody to label the receptors and computer assisted image analysis to quantify the bound antibody to combine the objective quantification of ligand binding analysis with the specificity of immunohistochemistry.

Chapter 4:
Breast tumour cell invasion and EGF receptor

Section i: Review of evidence of EGF receptor mediated metastasis

There is good evidence that breast tumours with cells expressing higher levels of EGF receptor (reviewed in Klijn 1992) and tumours with concomitant high EGF content and high EGF receptor expression (Karameris 1993) are associated with poor prognosis. However, the mechanism of this prognostic influence remains unclear.

Death from breast cancer is not usually the result of locally aggressive disease but rather occurs due to the development of metastatic disease. This raises the possibility that EGF receptor exerts its prognostic effect via an influence on metastasis. There is some evidence to support this hypothesis: the gene coding for EGF receptor is commonly amplified in glioblastomas but is not so in encapsulated gliomas (Schlegel 1994, Collins 1993); invasive bladder cancers express higher levels of EGF receptor than non-invasive counterparts (Neal 1985, Nguyen 1994); breast cancer patients whose tumours have elevated levels of EGF receptor more frequently have lymph node metastasis and reduced survival (reviewed in Klijn 1992, Sainsbury 1987). Metastasis can be broken down into a series of events (reviewed in Fidler 1990), early amongst these is the cancer cells' acquisition of an invasive phenotype. Part of this thesis explores the possibility that EGF receptor has a role to play in tumour cell invasion.

Carcinomas develop from epithelial tissues which are separated from adjacent stroma by a basement membrane layer (Vracko 1974). In normal tissues basement membrane forms a continuous barrier to movement of macromolecules and cells (reviewed in Liotta 1986). This remains true of benign pathologies, including those characterised by epithelial disorganisation and proliferation, however, adjacent cancers of epithelial origin this barrier becomes resorbed (Burtin 1982, Siegal 1981). Basement membrane is, therefore, the first physical barrier to malignant cells and invasion through this layer is a pathological prerequisite of carcinoma (Liotta 1984). The cancer cells' ability to invade this layer is clearly of crucial importance; after escape from the primary tumour, malignant cells must interact with host basement membranes at various stages of the metastatic cascade - entry to, and exit from, blood or lymphatic channels and invasion of distant organs. For these reasons a great deal of attention has focused on the genetic events that lead to the tumour cell phenotype responsible for invasion of basement membrane.

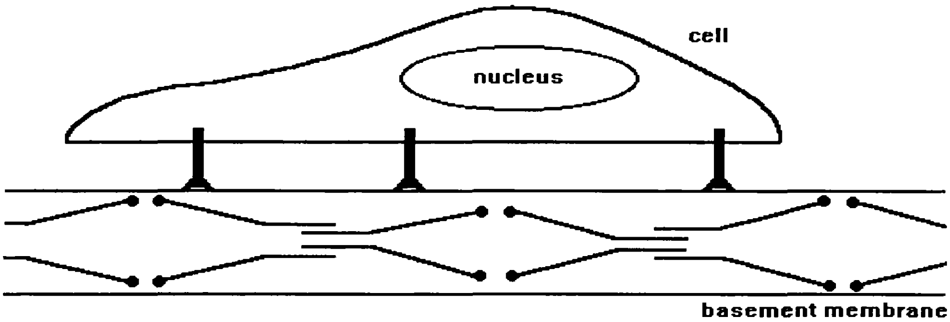
The principle components of basement membranes are laminin, collagen IV and a heparan sulfate proteoglycan (Timpl 1979, Laurie 1982). These molecules bind to one another and form an integrated supra-molecular structure of 5-10nm cords (Inoue 1983). Ultrastructural, chemical and biologic analyses indicate that this structure forms a physical sheet which resists the penetration of cells (Liotta 1986). To effect tumour cell invasion a cascade of enzymes must degrade the various components that together comprise basement membrane (Reich 1988). Cell surface expression of these enzymes plays a critical role in this degradation process (Stroppelli 1986). The potency of this local process, mediated by cell bound enzymes, is testified to by the fact that trypsin and bacterial collagenase have much less degrading effect (Parish 1992).

The process of tumour cell invasion is summarised in Figure 4.1.

In-vitro evidence suggests that ligand binding of the EGF receptor induces an invasive response which is independent of a similarly induced mitogenic response (Chen 1994). Also, ligand activation of EGF receptor has been shown to signal production of proteolytic enzymes (Yoshida 1990, Matrisian 1990), proteins modifying interaction with extracellular matrix (Thorne 1987, Lichtener 1993), and to enhance cell motility (Chen 1994, Chen 1994[1]). *In-vivo* evidence is less compelling.

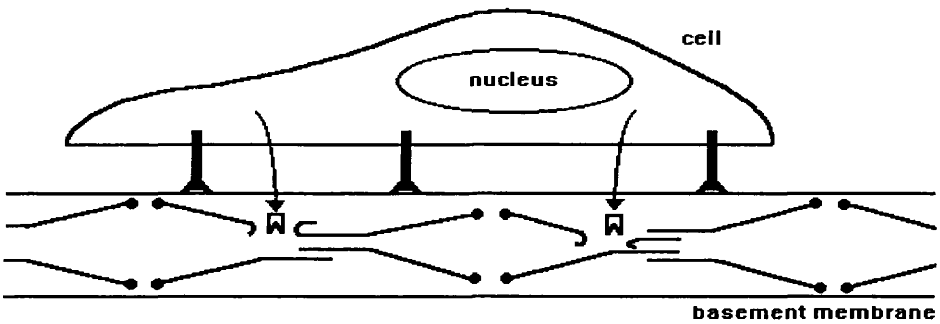
Sequential interaction of neoplastic cells with tumour neo-vasculature is believed to be one of the most significant steps in metastasis (Rice 1988). In a study of 165 patients, with median follow-up of 51 months, an analysis of EGF receptor levels added to the discriminatory power of a measurement of tumour angiogenesis in determining outcome; predicting disease free survival, in all women and in node positive women (Weidner 1994). A similar finding, but in node negative patients, has also been described (Fox 1994[1]). In these studies, EGF receptor's prognostic power could derive from receptor mediated modulation of tumour cell invasion into tumour neovasculature. Adding to this evidence is a study of the cell adhesion molecule E-Caherin. Tumour cell detachment form the primary lesion is an important step in the process of invasion (Coman 1947) and E-Cadherin has a critical role in initiating and maintaining cell to cell adhesion in epithelia (Takeichi 1988). Walker *et al* (1996) were able to demonstrate a significant

Step 1: Attachment



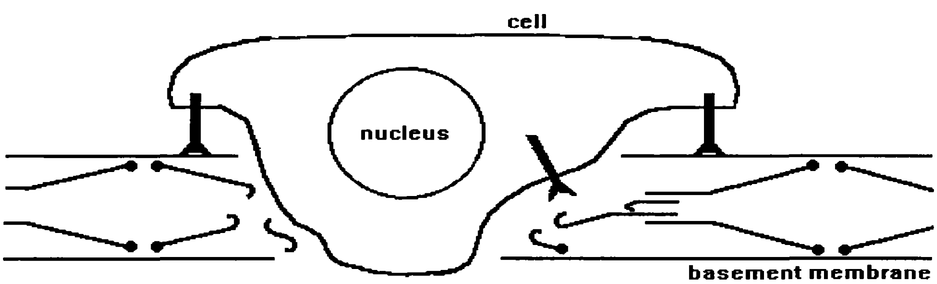
Cell attachment to the basement membrane. This is probably mediated by specific attachment factors which form a bridge between cell surface and elements of the basement membrane matrix.

Step 2: Degradation



Local degradation of matrix by tumour cell associated proteases. Localisation may be achieved by the amount of cell produced active enzyme out-balancing the natural protease inhibitors of the basement membrane matrix.

Step 3: Locomotion



Tumour cell locomotion into the region of matrix modified by proteolysis. The direction of locomotion may be modified by chemotactic factors.

Figure 4.1: Three-step hypothesis of tumour cell invasion. Schematic diagram, not to scale, modified from 'Tumour invasion and metastasis - role of extracellular matrix: Rhoads memorial lecture' Liotta LA, Cancer Res 46, page 2, 1986.

relationship between reduced levels of E-cadherin and EGF receptor expression and between reduced membrane expression of the former and lymph node metastases. Therefore, it could be postulated that EGF receptor stimulation induces tumour cell detachment. These citations provide only indirect evidence that EGF receptor influences tumour cell invasion, but such an influence could account for the prognostic significance of EGF receptor expression.

Direct evidence, that up-regulated EGF receptor signalling leads to increased tumour cell invasiveness, is scarce. One report is of a prostate cancer model (Xie 1995). Cells of the human prostate carcinoma cell line, DU-145, were transfected with either wildtype (WT) EGF receptor or a truncated, mitogenically active but motility deficient, EGF receptor. The truncated receptor lacked the carboxy terminus of the EGF receptor, including all its auto-phosphorylation sites. Ligand binding of this receptor has been shown to produce a full mitogenic signal but the receptor fails to internalise (Chen 1994). The parental DU-145 line produces EGF receptor and also the EGF receptor ligand TGF-α. Increased invasion occurred in the WT transfection and reduced invasion in the cells transfected with truncated receptor. The latter, it was postulated, resulted because of down regulation of the parental cell lines expression of EGF receptor. Monoclonal antibody, inhibitory to the EGF receptor, reduced invasion to a similar level for WT transfected, parental and truncated transfected lines.

Work using a colorectal model lends further support to the argument. The cell line, AA/C1, derived from a colonic adenoma, is clonogenic but not metastatic. From this parent line a metastatic variant has been produced, AA/C1/SB10. Brunton has reported (Brunton 1997) that EGF can stimulate invasion of this line, but not the parent line, into a Matrigel barrier. The invasive phenotype was associated with increased EGF receptor expression. This model is of particular interest as the adenoma to invasive carcinoma sequence in the progression of human colorectal neoplasia is well established and there are parallels with breast neoplasia, where carcinoma in-situ is thought to progress to invasive carcinoma.

The precise relationship between EGF receptor and outcome in breast cancer remains unclear, as is any causal link between its stimulation and the metastatic process. However, it would seem reasonable to speculate that, in human breast cancers, activation of EGF receptor, possibly by an autocrine loop, might promote tumour cell invasiveness and that this could be, partly or wholly, responsible for the poor prognosis of patients whose tumours have higher levels of this receptor.

Section ii: Review of models of breast cancer invasion

Since the time course of breast cancer evolution is typically 5-30 years, epidemiological protocols to study progression from in-situ to invasive disease would span 10-15 years (Holt 1993). Without considering ethical implications, this precludes meaningful or reproducible human studies and emphasises the need to develop appropriate experimental models of human breast cancer. However, development of systems which accurately reflect human disease poses difficulties.

Use of animal models is limited (Taylor-Papadimitriou 1993, Weaver 1995); cell lineages will vary from those of human disease and the molecules functioning in the biology of benign and malignant disease in study animals will show species variation. Nude mouse models are limited by the difficulties of growing human tumours in these animals compounded by the fact that those that will grow are frequently not representative of human disease (Fidler 1990). Transgenic mouse experiments circumvent the former of these problems but the tumours are of mouse origin. Whilst some investigators are attempting to genetically engineer these tumours to be more representative of human disease it is unclear how successful these efforts will be. A further concern with the use of murine models is that mouse breast anatomy is dissimilar to that of the human mamma (Ronnov-Jensen 1995).

Beyond the difficulties outlined above is the problem of interpreting the results of *in-vivo* studies. The individual steps, that together constitute the metastatic process, cannot be isolated, therefore the responsible genotypic and phenotypic changes cannot easily be elucidated. These difficulties make a cogent case for establishing *in-vitro* assay systems to study human material.

a) In-vitro invasion assays

A variety of increasingly refined assays have been developed to assess tumour cell invasion. These include chick heart models (Mareel 1979), urinary bladder (Hart 1978, Poste 1980), blood vessels (Poste 1980), lens capsule (Starkey 1984), chick chorioallantoic membrane (Poste 1980, Ossowski 1980) and the human amnion (Liotta 1980, Mignatti 1986).

The most commonly used of these has been the human amnion assay. There are, however, a number of problems with this technique: difficulties obtaining fresh samples after delivery, differences in membrane thicknesses and consistency, evaluation of micro-tears in the amnion, problems associated with radiolabelling tumour cells or matrix, variations in culture conditions for assays and anxiety of handling potentially blood contaminated samples when isolating the amniotic membrane (Basson 1985, Mignatti 1986, and reviewed by Hendrix 1987).

Each of the alternative assays also has disadvantages. The lens capsule assay, whilst using sturdier basement membrane, that is easier to manipulate than amnion, retains all of the disadvantages of an inconsistent biological membrane. Further, invasion is measured as an increase in permeability, thereby assessing membrane resorption and not strictly invasion or metastasis (Starkey 1984). In the chick embryo assay, metastatic load, determined by assay for human protein within the embryo, is used as a measure of invasion; separating the invasive component from a proliferative one is therefore difficult. Additionally metastatic mass varied with the mass of inoculum and embryo age at inoculation (Ossowski 1980).

All these systems make use of biological tissues. This is considered to be an advantage by some authors (Starkey 1984) since it may provide a better reflection of events *in-vivo* than systems which rely on artificial barriers. However, it also introduces problems; biological tissues are not uniform and this can lead to problems with data interpretation and reproducibility (reviewed in Hendrix 1989). Also, methodologies for these assays are complex and time consuming. These difficulties, and the fact that there are strong correlations between a cell's ability to degrade collagen IV, its invasive capacity *in-vitro* and its metastatic potential in nude mice or syngeneic hosts (Liotta 1986, Liotta 1991), have led to the development of reconstituted basement membrane assays.

b) Reconstituted basement membrane assays

Reconstituted basement membrane assays allow study of a specific part of the metastatic process - tumour cell invasion. This has caused them to be described as reductionist by some authors, however, they do provide some advantages over the biological barrier methodologies. Hendrix compared human amnion assays to reconstituted basement membrane assays (Hendrix 1989) and found that:

1. the measurable invasion in the former was less; human amnion is architecturally complex compared to reconstituted basement membrane,
2. invasion, measured in the latter, was considerably less variable; relating most probably to the variable thickness of the amnion barrier (Hendrix 1985).

Similar concerns have been mooted by other authors (Cresson 1986). A further advantage of reconstituted basement membrane assays is that the invasive cells can be recovered for further study. Thus, whilst not entirely replaced, the initial biological barrier techniques have been largely superseded by the reconstituted basement membrane assays.

The use of a compressed disc, comprising Laminin and collagen types I and IV, as basement membrane substitute to assess tumour cell invasion, was first described in 1986 (Terranova 1986). Concomitantly Kleinmann reported the development of Matrigel as a reconstituted basement membrane (Kleinmann 1982, Kleinmann 1986). Matrigel has a similar composition to the basement membrane associated with vascular and lymphatic conduits and thus provides a good means of studying tumour cell intravasation and extravasation. In the following year, there was the first description of the use of a contained *in-vitro* assay system for the study of the invasive potential of tumour cells; this incorporated both Matrigel, as a basement membrane substitute, and a filter barrier. (Albini 1987).

In this first assay, polycarbonate filters were coated with Matrigel, study cells were then placed above this filter, with chemoattractant below, and, after a period of incubation, invasion was determined as the number of cells that had crossed the filter. Subsequent investigators have maintained these basic features but have introduced a myriad modifications. Some of the features which have varied are the barrier mass, its preparation and the incubation period. Other basement membrane substitutes have also been described, but Matrigel remains the one with which greatest experience has been gained. The method of assay analysis has also undergone frequent modification, possibly reflecting lack of a satisfactory method of measuring invasion.

Section iii: Measuring invasion

Accurate measurement of invasion, in *in-vitro* Matrigel systems, is recognised to be difficult (Mackinnon 1992, Hendrix 1989, Parish 1992). As a result a large number of different methods have been reported; these include counting the mean number of cells crossing the filter in random optical fields (Albini 1987) and counting all cells that cross the filter (Hendrix 1989, Mackinnon 1992). These methods are disadvantaged in that they are tedious, may be subjective, and are prone to error; MacKinnon *et al* (1992) report results where the standard deviation frequently approaches the mean value despite multiple repetitions of their experiments.

Indirect methods of measuring tumour cell invasion have also been employed. These techniques include measurement of tumour cell degradation of the Matrigel barrier by quantifying radiolabelled molecules crossing the filter (Repesh 1989, Parish 1992). This incurs the inherent safety problems of working with radioactivity, also background radiation can make quantifying small invasive responses very difficult, and perhaps for this reason, the technique has been shown to offer no improvement on microscopic evaluation of invasion (Hendrix 1989). Matrigel degradation has also been determined using fluorescent dextran (Parish 1992). Proponents of these indirect methods of measuring invasion argue that they are reproducible and superior to cell counting as they focus on a single event in the metastatic cascade; basement membrane degradation. Others question the validity of this measure of tumour cell invasiveness, arguing that tumour cells can invade Matrigel barriers without producing significant degradation of the Matrigel layer (Noel 1991). Equally, since cell migration is necessary for tumour cell invasion measurement of Matrigel degradation may not correlate with cell invasion (Jones 1980).

Colorimetric methods overcome some of these difficulties. The most widely reported method employs 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Mossmann 1983). This chemical is converted to a dark blue pigment, formazan, by mitochondrial dehydrogenase, the absorbance of which can then be measured using spectrometry. Its application to Matrigel assay systems was pioneered by Schlechte (1990) and later modified by Imamura (1994). This technique has a number of potential limitations. Only living cells will produce formazan, with longer assay times some cells crossing the filter may die and these will not be counted using this method. Non-invading cells must be removed prior to treatment with MTT and this may include cells which have entered the Matrigel layer but have not crossed the filter (Imamura 1994). Further, in our hands removing non-invading cells from the filter surface, in 6.5mm inserts, proved technically demanding and could not be achieved with confidence. A final consideration is that no information regarding cellular morphology is acquired.

Section iv: Using confocal microscopy to measure invasion

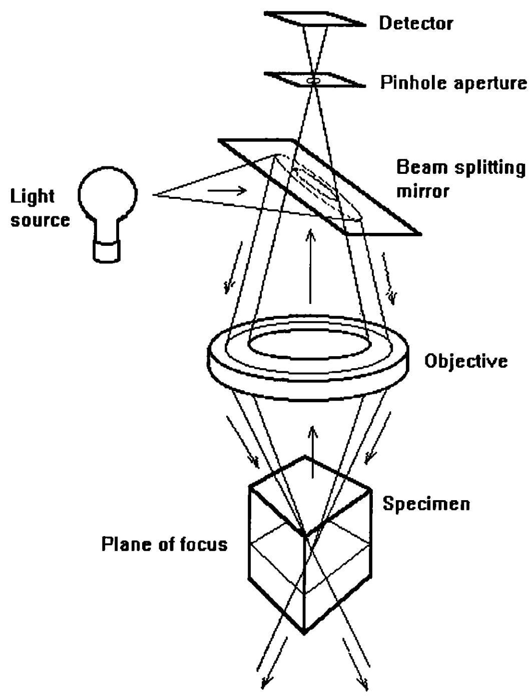
In the year prior to commencement of this study, Dr B Ozanne's group at the Beatson Institute for Cancer Research, Garscube, had developed a modification of the Matrigel/Boyden chamber assay. This used a confocal microscope to measure tumour cell invasion.

Confocal microscopy was invented by the father of artificial intelligence, Marvin Minsky (Lichtman 1994). Minsky wanted to map neuronal connections but was aware that cutting thin sections of neural tissues introduced the technical difficulties of producing thin sections and problems of interpreting three dimensional structure. Imaging the depths of thick sections, using conventional microscopy, posed two main problems. To obtain a high resolution image of a single plane, ideally light from this plane alone should return to the detector. Using conventional microscopy light from planes above and below the plane of interest returns to the detector leading to blurring. The second problem is scatter, which causes reduction in contrast with light caroming randomly from particle to particle prior to returning to the detector. This scattered light conveys no meaningful information and confuses the light signal from the plane of interest.

Confocal microscopy overcomes both these problems. The illuminating light is focused into an hour-glass shaped beam, a point in the plane of interest is illuminated by the most intense part of this beam, that at the 'waist', and this reduces the scatter effect. By interposing a pin-hole aperture between reflected light and the detector most of the light from planes above and below that of interest can be excluded, thus reducing blurring (White 1987, Wilson (editor) 1990). This arrangement provides an image of a single focus point within a thick section. To acquire an image of a plane the focus point must move over the section; this is scanning confocal microscopy. The development of lasers, which can be focused precisely, and of computer assisted image processing has meant that confocal microscopy has come a long way since Minsky's original work.

Dr Ozanne's group realised that computer assisted laser-scanning confocal microscopy could be used to accurately analyse stacked optical sections of the fragile Matrigel layer without its disruption. This overcomes many of the problems eluded to above: measurement would be of cell invasion not barrier degradation; computer assisted analysis could provide accurate quantification whilst overcoming the tedium of conventional measurement methods; since cells would be visualised morphology could be assessed and cells would not need to be alive to be detected.

Figure 4.2 : Schematic diagram demonstrating the principles of confocal microscopy



Light is focused by the objective lens to illuminate maximally the point of interest in the specimen. Light reflected from this point is focused so as to pass through a pinhole aperture to reach the detector. The pinhole prevents light reflected from other points (above and below the plane of focus) in the specimen from reaching the detector. By rapidly moving the light an image of the plane of focus is obtained. In the diagram, the outer cones represent light from the light source, the inner cones are reflected light from a point in the plane of focus. The arrows indicate the direction of light travel.

STATEMENT
OF
AIMS

The introduction to this thesis reviews the biology of the EGF receptor and implies a role for it in breast cancer. It could be summarised as reporting that patients whose breast cancers express higher levels of EGF receptor have a poorer outcome, but an accurate understanding of this relationship, and its mechanism are not clear. This results from;

- The lack of an accurate method of measuring expression of EGF receptor in breast cancers.

- An imprecise understanding of the consequences of receptor stimulation.

This thesis examines these problems.

The specific aims may be stated as:

- To establish levels of EGF receptor expression in a series of primary breast carcinomas using a quantitative method - radioimmunohistochemistry.

- To assess the validity and reproducibility of this method by comparing it to conventional measurement techniques.

- To determine the significance of receptor density, so measured, in terms of its relationship to traditional prognostic factors and to clinical outcome.

- To address the prognostic mechanism of the EGF receptor; by exploring the relationship between receptor expression and tumour cell invasion.

PART 2

MEASURING EGF RECEPTOR USING RADIOIMMUNOHISTOCHEMISTRY

Introduction to the experimental parts of the thesis

Two distinct sections of experimental work address the aims of this thesis. The first attempts to examine EGF receptor measurement and its relationship to clinical variables including outcome. In the second, a novel invasion assay is established and is then used to study the relationship between EGF receptor and invasion.

Overview of receptor measurement studies

Receptor was measured accurately in a panel of greater than 200 primary breast cancers using a radioimmunohisochemical technique. To confirm the validity of this measurement method, a proportion of these tumours were also analysed by ligand binding and conventional immunohistochemical techniques. The results of the techniques, along with the techniques themselves, are then discussed.

Clinical data, for the tumours studied using radioimmunohistochemistry, were collected into a database. The aims of this section were completed by determining if the accuracy of the radioimmunohistochemical method imparted a clinical advantage.

Parts of this experimental work have been published in the paper 'Quantitative estimation of epidermal growth factor receptor and c-*erb*B-2 in human breast cancer' Robertson KW, Reeves JR, Smith G, Keith WN, Ozanne BW, Cooke TG and Stanton PD, Cancer Research 56:3823-3830,1996

Chapter 5:
EGF receptor measurement

Section i: Tissues available

Tumour tissues, from patients with breast cancers treated surgically at Glasgow Royal Infirmary between 1984 and 1994, were used for these studies. Tissues identified macroscopically as tumour, either in theatre or in pathology immediately after resection, were taken into and stored in liquid nitrogen (-70°C). To confirm the presence of tumour, 5μm cryostat sections, cut and stained with Haemotoxylin and Eosin (H&E), were examined using light microscopy. Those tumour pieces which did not contain significant tumour tissue were discarded.

Sections for both the radioimmunohistochemical method and for conventional immunohistochemistry were cut at the same time. The ligand binding method was performed some time after the others. It has been reported (Koenders 1991) that at lower membrane protein concentrations the ligand binding technique may lose sensitivity. Therefore, to ensure that an adequate amount of membrane protein would be available for study, only tumour sections of 200mg or greater were used for the ligand studies reported here.

The radioimmunohistochemical method was applied to 203 primary breast cancers. Fifty of these tumours were studied using ligand binding and conventional immunohistochemistry.

Section ii: Radioimmunohistochemistry

This is a quantitative immunohistochemical method that was pioneered in squamous tumours by Fred Hendler in Dallas and latterly Louisville (Hendler 1984). The technique uses a radiation detection system, quantified by emulsion autoradiography. Under the auspices of Professor P Stanton and Dr B Ozanne, this methodology has been established in the Department of Surgery at Glasgow Royal Infirmary (Stanton 1994). The technique, as applied to breast tumours, is given in detail in the following section.

a) Antibody

The antibody used for these experiments was the EGFR1 mouse monoclonal that was used for the conventional immunohistochemistry studies. This antibody was radio-idoninated by the iodogen technique (Harlow 1988); 15μg of EGFR1 was iodinated using 250μCi of ^{125}I (Amersham). Antibody bound iodine was separated from free ^{125}I by sephadex G25 gel filtration. Levels of activity of the iodinated antibody were of the order of 500MBq/mg (500 counts per second per ng of antibody). Activity at time of use was ascertained from a standard decay table for ^{125}I. Antibody was used not later than one month after iodination.

b) Incubation

For each tumour specimen three sections were studied, duplicate test sections and a negative control. 5μm thick frozen sections were cryostat cut onto silane coated slides and stored in airtight packaging at -70°C until use. A further sequential section was stained with H&E to confirm the presence of tumour.

Sections to be analysed were pre-fixed in absolute acetone for 10 minutes and then washed twice with PBS (10mM sodium phosphate, 140mM sodium chloride, pH 7.4).

Application of antibody followed pre-incubation with a 100μl volume of a 1:1 mixture of PBS and normal rabbit serum. This pre-incubation was conducted for one hour, after which, ^{125}I labelled antibody was added to each section and mixed by pipetting. The specific activity of iodinated antibody was adjusted with unlabelled antibody so that 50 ng (4 KBq) of iodinated antibody was added to the pre-incubation mixture in a volume made up to 10μl with 1:1, PBS : normal rabbit serum. Incubations were carried out for 3 hours in humidified chambers at 22°C, at the midpoint of incubation the solution on the slide was mixed by pipetting with a Gilson pipette. Sections were then washed through three 10 minute changes of PBS, fixed for 10 minutes in 2% formaldehyde and washed through 3 changes of distilled water before being air dried.

c) Film autoradiography

The slides were secured into an X-ray cassette and overlaid with X-ray film (Dupont Cronex) which was exposed for about 72 hours at 4°C. This film was used as a guide to the activity of each section, in order to determine how long to expose each trio of tumour sections once these were coated with radiographic emulsion. In this manner it was possible to ensure an appropriate grain density for counting. Details of this aspect of the assay are included in the section pertaining to grain counting.

d) Emulsion autoradiography

Autoradiographic emulsion, Kodak NTB-2 diluted 1:1 with distilled water at 43°C, was used to coat the slides. This procedure was carried out in a dark room with the use of a Kodak No. 2 safelight. Blank slides were dipped in the emulsion to ensure it was bubble free. The test slides were then dipped, stood on end to dry in perspex racks, and then placed in standard metal slide-staining racks. The slides were then stacked in a metal tin with freshly desiccated silica gel in its base. The tin was sealed, wrapped in a thick, black plastic bag, and stored at 4°C. It has been our practice to mount sections toward the end of the slide, thereby only a small part of the slide was immersed in emulsion to coat the section. The radiographic emulsion is the most expensive consumable item in this protocol.

The radiographic emulsion was exposed to the sections for a period determined by the autoradiographic film; highly expressing tumours and cell lines being exposed for 4 hours, the intermediate tissues for 24 and 48 hours and the weakly expressing specimens for 4 to 7 days.

The radiographic emulsion was then developed and fixed. In the darkroom, a waterbath was filled with ice/water slurry, and in this were placed 4 slide staining dishes, containing the developing and fixing agents. The ice/water slurry ensures the developer is kept at approximately 10°C. After the appropriate duration of exposure, slides were retrieved from the sealed tin under darkroom conditions and immersed, sequentially, in each dish as below.

dish 1	Kodak D 19 developer (1:1 in distilled water)	4 minutes
dish 2	distilled water	1 minute
dish 3	Kodak Unifix fixer	5 minutes
dish 4	distilled water	1 minute

Slides were then thoroughly washed for 20 minutes through four changes of distilled water.

e) Counterstaining

Sections were stained 'through' the emulsion with 0.12% Safranin O for 5 minutes before being washed in tap water and mounted in DPX. Safranin (Basic Red) is a red nuclear dye, its use allowed sections to be oriented by comparison with the sequential H&E section. It is however difficult to use. Even when stored with acetate buffer (9:1, 0.1M Na acetate : 0.1M acetic acid), its shelf life is short, maximum one month. Further, it was necessary to use longer staining intervals as the solution aged. The dye also washes out rapidly, and staining had to be brief and carefully monitored. Finally, staining must be light, otherwise the Safranin can produce sufficient opacity to appear as grains to the image analysis system. If light microscopy revealed the counterstain to be excessive, the coverslips were removed and the sections destained with water before re-analysis.

An example of the finished product, Figure 5.1, is shown overleaf.

f) Controls and standards

From each tumour three sections were studied, duplicate test sections and a negative control. The control section was incubated with a 100 fold excess of cold antibody (5µg/section) compared to hot (iodinated) sections. The cold antibody was added to the pre-incubation mixture, that is before its hot counterpart. Analysis of this section provided an estimate of non-specific binding.

With each batch of tumours, a set of standard sections was tested; normal breast from a reduction mammoplasty and 5µm sections of cell line pellets, including A431 (produced as described in the ligand binding studies section of this chapter). These standards were processed in the same manner as tumour sections.

g) Grain counting

The radioimmunohistochemical method assumes that the density of silver grains developed in the emulsion layer reflects the amount of iodinated antibody bound to receptor, and that, in turn, this reflects the level of receptor expression. Saturating amounts of iodinated antibody are used to ensure the latter. The second assumption, that the emulsion will respond to ^{125}I disintegrations in a linear fashion with time, was confirmed by including two cell line pellet controls: the relative level of silver grains for the two controls remained constant for the range of exposure times required for the tumour specimens (the effect of ^{125}I decay was ignored given that the maximum exposure period was one week).

A Joyce-Loebl MiniMagiScan image analysis system connected to an Olympus OM-2 microscope was used for estimation of silver grain density. This process required that the operator outline areas of tumour in the section which was viewed with a 40x objective. Identification of tumour was facilitated by examination of the serial H&E section. The analysis system then calculated both the area outlined and silver grain density within the area. This process was repeated for at least ten

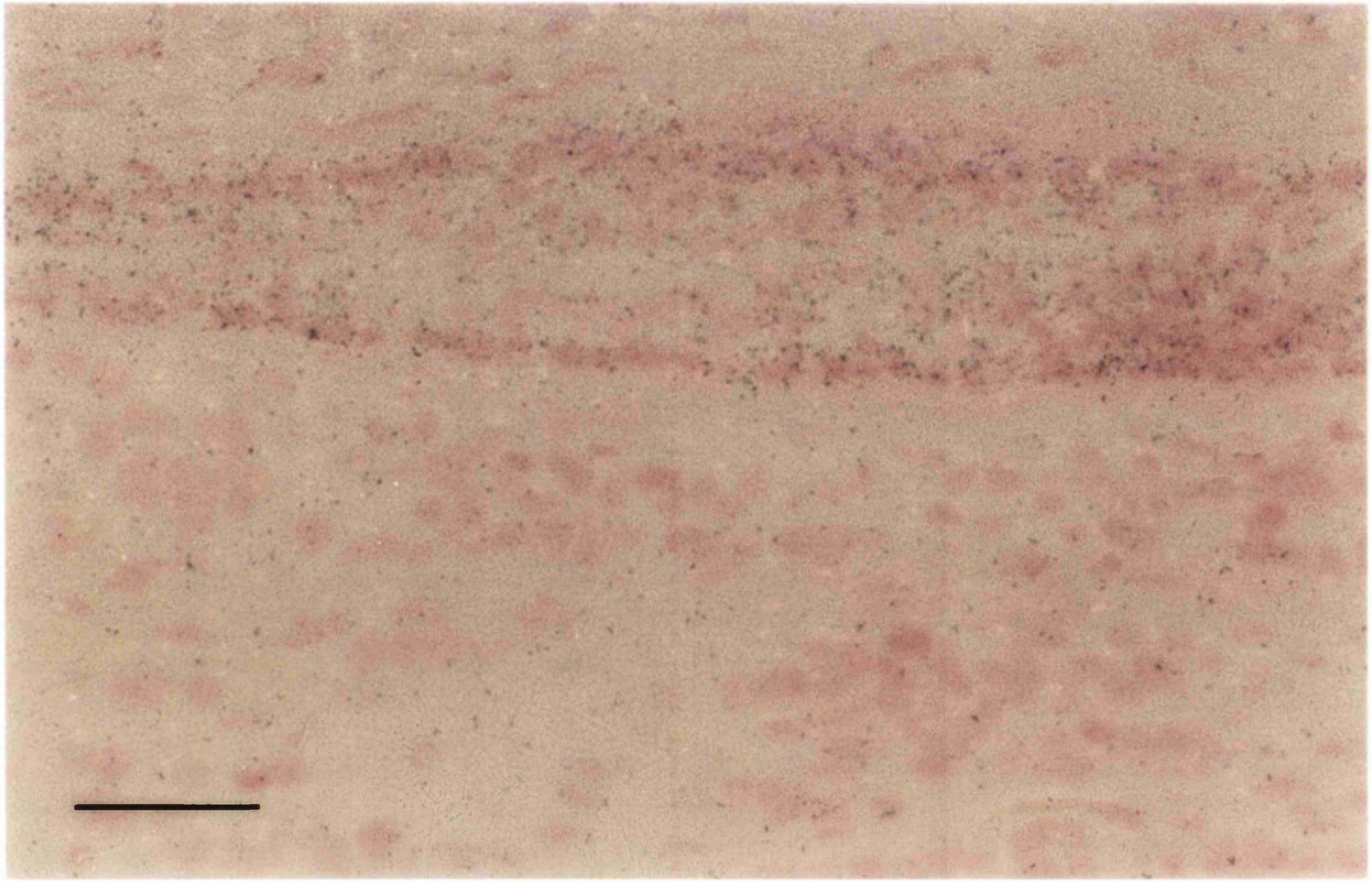


Figure 5.1: Photomicrograph of a breast tumour section following the radioimmunohistochemical protocol. The nuclei are counterstained red with safranin. The scale bar measures 50μm. Note the higher density of silver grains over the normal duct (top of section) compared to the tumour cells. Quantification is achieved by comparing the grain densities of the tumour section to those of cell line pellets with known receptor levels.

fields in any tumour section. Data was stored, by the system, for subsequent analysis.

The image analysis system worked by determining the optical density of each of the 512 vertical by 512 horizontal pixels which made up the optical field. If the optical density exceeded a certain threshold then the pixel was counted as positive. Since a silver grain could encroach on more than one pixel, the image analysis system was capable of identifying discrete objects, that is all touching 'positive' pixels were counted as though part of a single object. A thick emulsion layer might result in a number of overlapping silver grains, making grain counting difficult. To reduce this effect, the emulsion was diluted with water (1:1), allowing slide dipping to produce a thin emulsion layer. Thus there was an optimal range of grain density for counting and the film autoradiography stage of the protocol was included so that the investigator could make an estimation of the period of emulsion exposure required to achieve this grain density.

h) Calculation of receptor expression

Results were calculated as silver grains per unit area, these were converted to silver grains per unit area per hour of emulsion exposure, averaged over the 20 fields on the duplicate sections of each tumour. From this score was subtracted the counts (per unit area, per hour) for the negative control section, taken to represent non-specific binding. This final result was expressed as a % of the receptor expression for the cell lines, run as standards with each batch of tumours (see *Controls and standards*). Tumour results were subsequently converted to a % of counts for normal breast based on the average count in the 9 normal breast samples (tissue taken from reduction mammoplasty specimens).

Table 5.1 provides an example showing grain counts for duplicate sections of one of the normal breast specimens. Hot section indicates treatment with an excess of radio-iodinated antibody, two sections for each specimen, and the negative control is the cold section.

This example is for normal breast but results for tumour specimens were generated in a similar fashion. To allow results from different tumour batches to be directly compared they were expressed as a % of the measure of the A431 grain counts for the A431 section included in that batch. A panel of 9 reduction mammoplasty specimens were also studied using the radioimmunohistochemical method, by using the mean value of these results (range: 5.28 - 9.70% of A431 levels), it was possible to compare tumour EGF receptor expression with that in normal breast tissue.

Table 5.1: Calculating EGF receptor expression in normal breast.

slide	Area counted	Grains counted
Hot section 1	3.40	890
Hot section 2	2.94	946
Cold section	3.22	232

Total area of interest :	$3.40 + 2.94 = 6.34 \times 10^{-2}mm^2$
Total grain count :	890 + 946 = 1836
Emulsion exposure time :	17.5 Hrs
∴ For the hot sections, mean grains/$10^{-2}mm^2$/Hr :	1836/6.34/17.5 = 16.55
For the cold section, mean grains/$10^{-2}mm^2$/Hr :	232/3.22/17.5 = 4.12
∴ For this section, net grains/$10^{-2}mm^2$/Hr :	16.55 - 4.12 = 12.43

Section iii: Ligand binding

a) Reagents

The protocol used for these experiments was based on that advocated by the Commission of the European Communities; 'Detailed methodologies of the development of assays for growth factors and growth factor receptors' Leake, RE, Foekens JA and Benraad TJ. Commission of the European Communities, EUR14799EN, 1993. The reagents used are those advocated by this protocol unless otherwise stated.

Buffer A

This buffer was a 0.01M phosphate buffer with a pH of 7.4. The final buffer solution comprised; 0.01M K_2HPO_4, 0.01M KH_2PO_4, 0.0015M Disodium EDTA, 0.003M NaN_3, 0.01M monothioglycerol, 10% glycerol (v/v) (Leake 1993).

Buffer B

This was a 0.02M phosphate buffer with a final pH of 7.4. It comprised; 0.02M K_2HPO_4, 0.02M KH_2PO_4, 0.15M NaCl and 70μg/ml Bacitracin (Koenders 1991).

HAP slurry

The EORTC Receptor Study Group has proposed that the hydroxyapatite (HAP) assay be used as standard for measurement of EGF receptors in human breast cancers (Benraad 1990). BIO RAD DNA grade Bio-Gel® HTP was used. This reagent has found a variety of uses in fractionation and purification processes (Gorbunoff 1984, Gorbunoff 1984[1]). HAP absorbs EGF receptor protein when the HAP-slurry is prepared in a Bacitracin-containing buffer. In this manner, EGF receptor bound ^{125}I-EGF can be separated form unbound ^{125}I-EGF by low speed centrifugation. Prior to the use of HAP, this separation had generally been achieved using high speed centrifugation (10 000 - 100 000 g). The slurry was made as described by Benraad *et al* (1990); the dry HAP powder is mixed with buffer B so as to produce a product which is buffer/HAP, 3:2, volume for volume.

EGF / ^{125}I-EGF

Mouse epidermal growth factor was used. This ligand was labelled using the iodogen method (Harlow 1988). The authors of the European Communities Commission report (Leake1993), suggested that this method could cause oxidation of EGF with resultant non-equivalent binding behaviour of labelled ligand in comparison to unlabelled ligand. They advocated the iodine monochloride method (two other satisfactory methods were described but these required reagents that have subsequently been withdrawn from the market). There are, however, a number of reports of EGF receptor expression rates in breast cancers which have utilised a ligand binding assay with EGF iodinated using the iodogen method. Our laboratory has had considerable experience of the iodogen method but none with the iodine monochloride technique. The latter technique is also technically more demanding and expensive. For these reasons the iodogen method was used for this series of experiments.

This part of the procedure was performed in a lab fume hood specifically designated for radioactive work. Briefly, 2μg of the mouse-EGF in phosphate buffered saline (PBS) was added to an Iodogen coated tube. 250μCi of ^{125}Iodine (^{125}I) (Amersham) was then added and the reactants were incubated for 5 minutes. By adding excess of protein, in this case foetal calf serum, the reaction was stopped. A Gel filtration Sephadex G25 column (NAP-10) was then used to elute the ^{125}I bound EGF. The column effects this separation as the larger molecule ^{125}I bound EGF passes through the column more quickly than low molecular weight unbound ^{125}I. Radio-iodinated EGF was stored in aliquots under suitable radioactive shielding at -20°C.

It was assumed that the first peak of activity eluted from the Sephadex G25 column contained only iodinated EGF. The specific activity of this fraction was determined using a gamma-counter. Assuming 80% counting efficiency, counts per minute were converted to μCi for use in the LIGAND program.

Care was taken to ensure that contaminated materials were disposed of in a safe manner and appropriate paperwork for handling of radioactive materials was completed.

b) Obtaining the membrane component

The protocol assays membrane bound EGF receptor and therefore the membrane component of the homogenate must be separated from the remainder. Tumour pieces were retrieved from the liquid nitrogen tumour bank and presence of tumour confirmed as described above.

- In a universal container, the specimen was immersed in approximately 1.5ml of Buffer A.
- Using an Ultraturrax, with 3 x ten second bursts of maximum speed, specimens were homogenated.

Throughout this part of the procedure tissues were kept on ice and between each specimen the Ultraturrax blade was cleaned with alcohol.

- The resultant homogenates were centrifuged at 500rpm for 5 minutes at 4°C, this 'pelleted' the heavier nuclear debris.
- Membrane containing supernatant was decanted to eppendorfs, the remainder was discarded.
- Membrane component was then retrieved by ultracentrifuging the eppendorfs at 15 000rpm for 30 minutes at 4°C.
- Supernatant was discarded and the 'pelleted' membrane component was resuspended in Buffer B to a total volume of 1.25ml.

c) Protein estimation

A modification of the spectrophotometric method described by Bradford (1976) was used. This technique is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G250 shifts from 465nm to 595nm when protein binding occurs.

- 100μl of each membrane suspension was made up to 2ml with Buffer B.
- From this, 1ml aliquots of 1:2, 1:5, 1:10, 1:50 and 1:100 dilutions were made using Buffer B.

- In a Rohen tube, 800μl volumes of each of these dilutions was added to 200μl of BioRad protein estimation reagent.
- After vortexing twice and decanting to a cuvette, optical density at 595nm was measured.
- Bovine serum albumen (BSA) was used to make a panel of protein standards. 800μl aliquots of 0, 1, 2, 5, 10, 20, 50 and 100μg BSA in Buffer B were used.
- Protein concentration in the membrane suspension was estimated by comparing optical density readings for the tumour membrane with those obtained from the panel of BSA standards.

d) Sample storage

Using the protein estimation data, the remaining 1.15ml of membrane suspension was diluted to 1.1mg of protein/ml using Buffer B. To 1ml aliquots 100μl of 1.1% BSA (in Buffer B) was added as described by Benraad (1990). It has previously been demonstrated that these membrane suspensions can then be stored in liquid nitrogen for subsequent analysis without compromising results (Grimaux 1990, Koenders 1991).

e) Assay protocol

- Stored membrane suspension samples were retrieved and thawed.
- Samples were vortexed and split into ten 100μl aliquots in eppendorfs. For each sample there were 10 eppendorf tubes; tubes 1 to 8 were assay tubes, tubes 9 and 10 were control tubes.
- Added to these tubes was a 30μl volume of Buffer B with the ^{125}I-EGF concentration indicated in the table below.

tube	^{125}I-EGF (nM)
1	0.72
2	1.6
3	3.2
4	4.0
5	4.8
6	6.4
7	12.0
8	16
9 (control)	4.8
10 (control)	4.8

Table 5.2: Concentrations of ^{125} I-EGF in the ligand binding protocol.
To minimise pipetting error 3 stock solutions were produced; for tubes 1 and 2, for 3 to 6, 9 and 10, and tubes 7 and 8. A minimum pipetting volume of 10μl was used.

- To assay tubes (1 to 8) 10μl of Buffer B was added, to control tubes (9 and 10) an excess of unlabelled EGF; 10μl of 3.6mM unlabelled EGF producing a final assay concentration of 150nM in 240μl.
- Eppendorfs, all of which now contained a volume of 240μl, were incubated for 16-20 hours at 4°C.
- After incubation, 100μl of HAP slurry was added to each eppendorf.
- Eppendorfs were incubated, at 4°C, for a further 1 hour during which each was vortexed 3 times.

- To pellet the HAP slurry eppendorfs were centrifuged at 800rpm for 2 minutes at 4°C. The HAP slurry trapped the membrane component including the membrane bound EGF receptor and any amount of ^{125}I-EGF bound to it.
- Supernatant was discarded and the pellet washed in 1ml of Buffer B. Eppendorfs were then centrifuged for a further 2 minutes at 800rpm at 4°C.
- This wash step was repeated three times.
- The tips were then cut from the eppendorfs into pony vials using a eppendorf tip guillotine. Care was taken to ensure the whole HAP slurry pellet was transferred to the pony vial and that the tubes, 1 to 10, were kept in sequence.
- Pony vials were transferred, in sequence, to a gamma counter.

The gamma counter used for these experiments was a Coulter machine which could be programmed to automatically assay the pony vials producing a print-out of the counts detected over a one minute period.

f) Non specific binding

Control tubes, 9 and 10, provided an estimate of non-specific binding. The unlabelled EGF competed, with its iodinated counterpart, to bind EGF receptor. However, the former was added to enormous excess (1.2mM), effectively preventing receptor binding by iodinated ligand (4.8nM). These tubes were assayed in a similar manner to those containing no unlabelled EGF (tubes 1 to 8).

g) Data Analysis

The binding data were analysed with the curve fitting computer program LIGAND (Peter Munson, NIH Bethesda, MD 20892, USA), version 2.3-March '88. This program offers many advantages over the often inadequate graphical methods of treating binding isotherms. These advantages and a description of the advantages of the features of LIGAND are discussed in detail by Munson (1983).

Briefly, LIGAND fits the data by weighted non-linear least squares regression to exact mathematical models representing the binding of ligand to receptor. In its simplest form, one ligand binding to one receptor with single affinity, the model fits three parameters to the data describing the affinity of the interaction, number of binding sites present and the level of non-specific binding. The program calculates values for each parameter and estimates the degree of accuracy to which they have been determined. As EGF receptor is known to exist in two affinity states in many tissues, the data were also analysed using the more complex two binding site model. A variance ratio test was used to determine whether this two-site model was superior to the simple one-site model (Munson 1980). For most tumours the single-site model was better and therefore this model was used for each of the breast tumour specimens and also for the tumour cell lines.

LIGAND has a facility for pooling results from several experiments. This meant that results from separate experiments could be combined. The program assumes that the affinity of the binding reaction is the same for each experiment and that the number of binding sites varies over and above any previous corrections for cell number or membrane concentration. The program introduces correction factors for each curve and common parameters are then used to describe binding in the pooled experiments. Data treated in this way can provide more accurate estimations of the parameter values than is achieved by considering experiments separately (Munson 1983).

h) Reference preparations

Human placental tissue, rich in EGF receptor, was used. This tissue had been stored in liquid nitrogen and was treated in a similar manner to the tumour tissues. In comparison to tumour specimens, large amounts of this tissue was available allowing a standard placental sample to be assayed with each tumour series. By this means results from each tumour series could be compared. Placental tissue expresses large amounts of EGF receptor and, to prevent ligand depletion, smaller concentrations of placental membrane protein suspension, than for the tumour samples, were used.

i) Tumour cell lines

The ligand binding assay was used to estimate EGF receptor expression in the breast cancer cell lines used for the invasion studies.

The tumour cell lines were grown to sub-confluence in large flasks. They were then harvested by scraping them from the plastic, trypsin was not used as this would have cleaved the EGF receptor binding site from the cell surface. Cells were then transferred from the culture flasks to universal containers in which they were centrifuged at 500rpm for 5 minutes at 4°C. The supernatant was aspirated and the universal container was then dipped in liquid nitrogen to snap freeze the tumour cell pellet at its base. Pellets were retrieved by sharply tapping the universal container, they were then stored in liquid nitrogen until their use. In the ligand binding protocol cell line pellets replaced the tumour sections. Several studies of each tumour cell line were made.

Section iv: Conventional immunohistochemistry

a) Antibody

The antibody used for these experiments was the EGFR1 $IgG2_b$ mouse monoclonal originally described by Waterfield *et al* (1982), provided by Dr B Ozanne of the Beatson Institute for Cancer Research. This antibody recognises an epitope in the extracellular domain of the EGF receptor. Receptor measurement in paraffin fixed tissues presents technical difficulties when expression levels are low, as is the case with EGF receptor in breast tumours, since the sensitivity of the assay can be insufficient (Reeves 1996). These studies were performed on frozen sections of tumour.

b) Method

Three step streptavidin-biotin-peroxidase (SABC) immunohistochemistry was performed on frozen sections. 5μm sections were thawed onto silanised slides, stored at -70°C in airtight packaging, and allowed to warm to room temperature before opening. The sections were fixed in 100% acetone and washed in PBS. As we had previously identified that endogenous biotin was a problem in some breast tumours this was routinely suppressed by incubating the sections with 50% egg white for 30 minutes (Reeves 1994). Further non-specific binding was inhibited with serum blocking solution (10 minutes), containing 25% human serum and 25% rabbit serum in PBS, this was then aspirated off and replaced with EGFR1 (1μg/ml) supplemented with 0.02% free biotin. After 2 hours, as the second component of the endogenous biotin block, the sections were washed and incubated for 30 minutes with biotinylated anti-mouse immunoglobulins at a 1:400 dilution (Dako Ltd., High Wycombe, Berks.). The third SABC layer (conventional streptavidin biotin peroxidase complex) was applied for a further

30 minute incubation. After washing, the peroxidase signal was developed with a 10 minute exposure to 0.07% $NiCl_2.6H_20$, 0.025% diaminobenzidine tetrahydrochloride, and 0.01% H_20_2, producing a black precipitate. Nuclei were counterstained red with safranin before dehydration and mounting through standard solutions. Parallel control sections were processed similarly, with the mouse monoclonal antibody being replaced with non-immune mouse IgG_{2b} at a concentration of 1µg/ml.

c) Scoring

All sections were scored by a single investigator (Dr J Reeves) who was blind to results of both ligand binding and radioimmunohistochemistry. The percentage of tumour cells falling into negative, weakly positive, and strongly positive categories was qualitatively assessed. The sum of (1 x % weakly positive) + (2 x % moderately positive) + (3 x % strongly positive) was used as a histoscore, with a maximum of 300.

Chapter 6:
Results of measurement studies

Section i: Radioimmunohistochemistry

a) Normal breast

Nine reduction mammoplasty tissue specimens were used as normal controls for comparison with tumour samples. This allowed EGF receptor levels to be expressed relative to the mean of levels in this normal tissue. Expression levels above or below this mean are reported as over or under expression respectively. EGF receptor levels in the normal specimens were expressed as a percentage of receptor density of the cell line standard (A431 cells, known to have $2x10^6$ EGF receptors per cell (Hendler 1984, Stanton 1996)) which was processed with each batch of tissue samples (see Appendix 2). The normal levels ranged from 5.28 to 9.70% with a mean of 7.6% of the A431 levels (see Appendix 2).

b)Breast cancers

Results of receptor estimation by radioimmunohistochemistry are summarised in the histogram (Figure 6.1). Median tumour receptor expression was 4.8% of normal breast levels (range, 0 to 707%). All but 5 (3%) of the tumours had lower levels of expression than control breast reduction sections. 91% had less than half the normal number of receptors, and 70% less than 10% of this value. Thus the majority of breast cancers showed levels of EGF receptor expression that were much lower than normal, and only a very small percentage showed levels of expression greater than normal. Results for each tumour are given in Appendix 2.

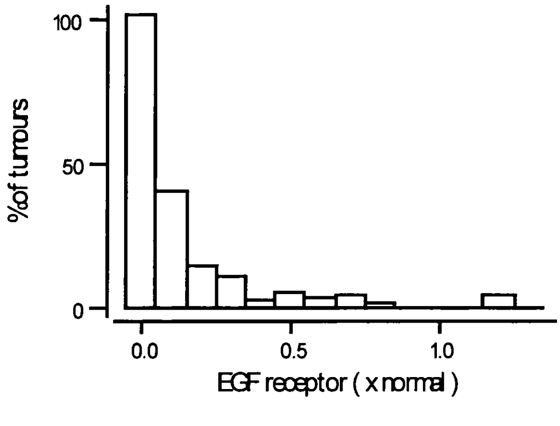


Figure 6.1: EGF receptor expression measured by radioimmunohistochemistry

Frequency histogram of EGF receptor expression in breast cancers. The results are expressed relative to the mean level in normal breast.

Section ii: Receptor measured by three methods

Fifty of the tumours, designated t1 to t50, were assayed for EGF receptor by each of the three measurement techniques; ligand binding, conventional immunohistochemistry and radioimmunohistochemistry. Breast cancer cell lines used in the invasion studies were also studied using the ligand binding and radioimmunohistochemical methods. Complete listings of the data from each method are included in Appendix 1.

a) Ligand binding studies

The mean membrane protein concentration was 792µg/ml. Using a similar assay, with the same membrane protein estimation technique, Koenders *et al* (1991) proposed a lower assay sensitivity threshold, of 200µg/ml. Of the 50 tumours in this report only two, t13 and t38, produced membrane protein concentrations less than this and one of these was determined to be receptor positive.

The LIGAND program used the binding data produced by the assay, along with estimations of non-specific binding, to produce a measurement of EGF receptor. Using this software, 19 (38%) of the tumours were EGF receptor positive (had measurable receptor levels), the range of expression was 1.6 to 472 fmol/mg membrane protein (mean 52.4 fmol/mg). The program was unable to fit a curve to the binding data for 31 (62%) of the tumours. For the purposes of this study these were considered EGF receptor negative. The results of each batch of tumours could be standardised using results for the placenta standard (see Appendix 1).

b) Conventional immunohistochemistry

All tumours were scored by a single investigator (Dr J Reeves) who was unaware of the results of either radioimmunohistochemical or ligand binding studies. Twenty four, 48%, of the tumours were scored as positive for EGF receptor. The mean histoscore in this group was 114, range 5 to 280. Twenty six tumours, 52%, did not stain for EGF receptor using this protocol.

c) Radioimmunohistochemistry

Results for all tumours analysed with this method are given in *section i* of this chapter. The following are the results for the 50 tumours analysed by all three methods. EGF receptor could be measured in all but 4 tumours, that is, in 92% of the tumour specimens. For tumours with measurable receptor, the range was 0.1 to 53.8% (mean 3.58%) of the grain counts for the A431 cell line.

Table 6.1: EGF receptor measurement in breast cancers

tumour	Lb (fmol/mg)	Cihc (histoscore)	Rihc (%A431)	tumour	Lb (fmol/mg)	Cihc (histoscore)	Rihc (%A431)
t1	136	0	0.1	t26	-	70	1.19
t2	23	0	0.39	t27	-	0	0.12
t3	-	0	0.17	t28	-	0	2.05
t4	-	0	0.48	t29	-	0	0
t5	32	135	5.5	t30	-	0	0.53
t6	-	96	1.98	t31	-	100	1.82
t7	-	0	0.13	t32	-	0	0.36
t8	-	0	0.12	t33	-	0	0.33
t9	-	0	0.44	t34	-	15	0.89
t10	-	100	5.4	t35	-	5	0.74
t11	-	0	0.31	t36	-	0	0.19
t12	-	0	0.21	t37	-	0	0.17
t13	-	0	0.19	t38	9	270	13.36
t14	-	0	0.32	t39	32	12	0
t15	-	0	0.52	t40	-	50	1.44
t16	31	120	3.08	t41	14	110	3.85
t17	-	0	0.41	t42	15	150	2.45
t18	-	0	0.36	t43	472	150	25.28
t19	-	0	0.33	t44	4	250	18.56
t20	14	59	1.48	t45	2	210	3.61
t21	5	0	0	t46	73	50	1.28
t22	11	0	0.39	t47	-	100	1.44
t23	-	0	0	t48	37	190	4.84
t24	79	280	53.8	t49	-	40	0.27
t25	3	82	1.77	t50	4	100	1.85

Lb refers to the ligand binding method, results are shown as femto-moles of receptor per milligram membrane protein. The symbol '-' means that the LIGAND program could not fit a curve to the data from the assay, these assays all produced a very low value for R1 indicating very low receptor levels. Conventional immunohistochemistry, Cihc, results are shown as a histoscore, defined in the protocol, giving a range of 0 to 300. Rihc refers to the results of the radioimmunohistochemical technique. These are shown as a percentage of the grain counts obtained for the A431 cell line.

Section iii: Statistical comparison of methods

Only the 50 tumours evaluated with all three measurement methods were considered. Using both conventional immunohistochemistry and ligand binding, EGF receptor measurement is usually dichotomised; positive or negative. Ligand binding results were coded as positive if the LIGAND program produced a measurable value for EGF receptor and conventional immunohistochemistry was considered positive if the histoscore was greater than zero. This dichotomy splits the tumours into roughly equal groups and may aid statistical analysis. However, the distribution of radioimmunohistochemical results offers no such simple dichotomy. Therefore, the results of this method were assessed as a continuum.

Results of conventional immunohistochemistry and ligand binding were compared using a Chi-square analysis, the results of which are shown below.

		immunohistochemistry negative	immunohistochemistry positive	
ligand binding	negative	22	9	
	positive	4	15	d.f. = 1, p = 0.0006

Mann-Whitney *U* tests, comparing the results of radioimmunohistochemistry with both ligand binding and conventional immunohistochemistry, are shown in the table below.

	n	median r-ihc score (%A431)	
ligand binding - negative	31	0.36	
ligand binding - positive	19	2.45	d.f. = 1, p = 0.0019
immunohistochemistry - negative	26	0.32	
immunohistochemistry - positive	24	1.92	d.f. = 1, p < 0.00005

As noted, the above analyses were performed with ligand binding and conventional immunohistochemical results evaluated as positive or negative. A better impression of the comparability of the techniques might be obtained by comparing measured values for each tumour by each measurement technique. The following plots provide a graphical impression of this comparison which is analysed statistically in the accompanying tables.

Conventional immunohistochemistry and ligand binding.

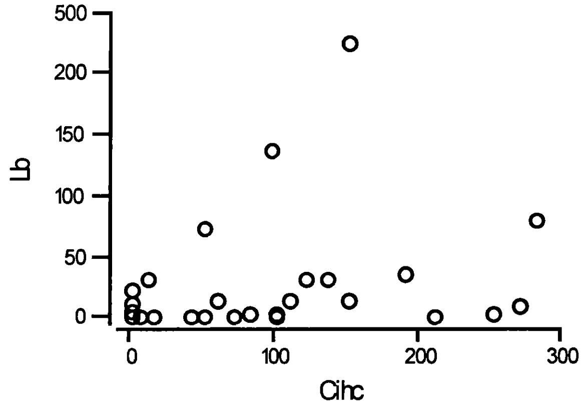


Figure 6.2 : Plot of ligand binding and conventional immunohistochemistry results. Ligand binding (Lb) results, in fmol/mg membrane protein, from the LIGAND program are plotted on the y-axis. Note there is a break in this axis, this allows a useful presentation of the data set whilst including the single very high result. Conventional immunohistochemistry (Cihc) is plotted on the x-axis using the histoscore as outlined in the conventional immunohistochemistry protocol.

This relationship was analysed by Spearman rank correlation analysis.

exclusion criteria	n	r2	p
none	50	0.312	< 0.0005
tumours with zero receptor by Cihc	24	0.208	0.025
tumours with zero receptor by Lb	19	0.002	0.869

Table 6.2: Comparison of conventional immunohistochemistry and ligand binding methods. The data were derived from a Spearman rank correlation analysis of the results of both receptor measurement techniques. The number of tumours analysed, n, was defined by the exclusion criteria indicated. r2 is the square of the Spearman rank correlation coefficient and p is the level of significance.

Radioimmunohistochemistry and ligand binding.

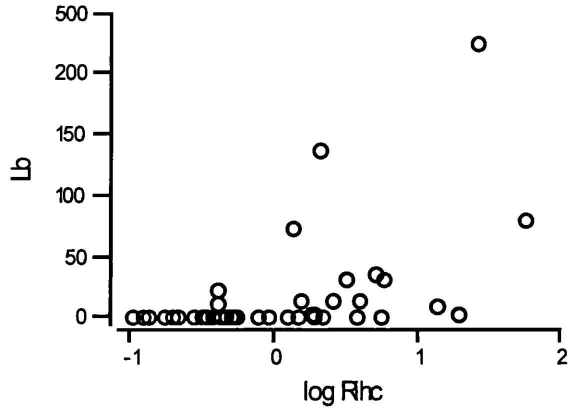


Figure 6.3 : Plot of ligand binding and radioimmunohistochemistry results. Radioimmunohistochemistry (Rihc) is plotted on the x-axis with tumour results, as a % of A431 grain counts, plotted on a $\log_{10}$ scale. Ligand binding (Lb) results, in fmol/mg membrane protein, from the LIGAND program are plotted on the y-axis. Note there is a break in this axis, this allows a useful presentation of the data set whilst including the single very high result.

As above, this relationship was analysed by Spearman rank correlation analysis.

exclusion criteria	n	r2	p
none	50	0.175	0.003
tumours with zero receptor by Lb	19	0.009	0.702

Table 6.3: Comparison of the radioimmunohistochemical and ligand binding methods. The data were derived from a Spearman rank correlation analysis of the results of both receptor measurement techniques. The number of tumours analysed, n, was defined by the exclusion criteria indicated. r2 is the square of the Spearman rank correlation coefficient and p is the level of significance.

Radioimmunohistochemistry and conventional immunohistochemistry.

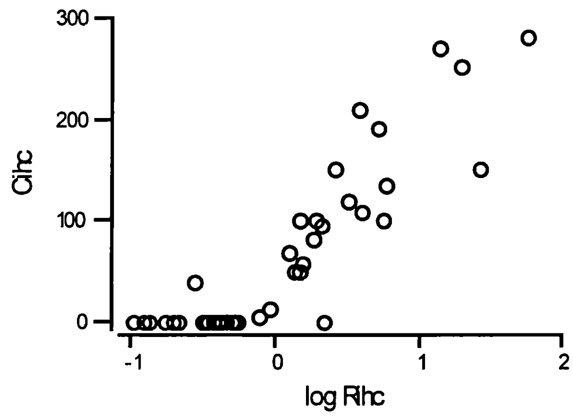


Figure 6.4 : Plot of conventional immunohistochemical and radioimmunohistochemical results.

The histoscore, defined in the conventional immunohistochemistry (Cihc) protocol is plotted on the y-axis. Radioimmunohistochemistry (Rihc) is plotted on the x-axis with tumour results, as a % of A431 grain counts, plotted on a $\log_{10}$ scale.

As above, this relationship was analysed by Spearman rank correlation analysis.

exclusion criteria	n	r2	p
none	50	0.695	< 0.0005
tumours with zero receptor by Cihc	24	0.838	< 0.0005

Table 6.4: Comparison of the radioimmunohistochemical and conventional immunohistochemical methods. The data were derived from a Spearman rank correlation analysis of the results of both receptor measurement techniques. The number of tumours analysed, n, was defined by the caveat described under the exclusion criteria heading. r2 is the square of the Spearman rank correlation coefficient and p is the level of significance.

Section iv: Results for the tumour cell lines

The cell lines used for the invasion studies (Part 3) were assayed using the ligand binding and radioimmunohistochemical protocols.

a) Ligand binding studies

	Protein (μg/ml)		Receptor (fmol/mg)		
cell line	Run 1	Run 2	Run 1	Run 2	combined
BT20	560	134	2340	4119	2628
BT474	440	114	513	1074	788
MDA-MB-231	420	420	1503	429	685
MCF7-ADR	460	92	389	2061	594
SKBR3	490	490	671	421	524
MCF7	780	780	5	-	29
MDA-MB-453	716	716	21	-	28
ZR75	1215	1215	19	12	25
MDA-MB-361	200	50	11	-	-

Table 6.5 : Ligand binding studies of the breast cancer cell lines
Protein (μg/ml) refers to the concentration of membrane protein in the assay volume. Receptor (fmol/mg) is the concentration of receptor per milligram of membrane protein as determined by the LIGAND program. The symbol '-' means that the LIGAND program could not fit a curve to the data from the assay, these assays all produced a very low value for R1 indicating very low receptor levels. The LIGAND program allows both sets of ligand binding data to be 'combined', using this technique produced the results in the column headed 'combined'.

Of the 9 cell lines, EGF receptor was measurable in 8. 'Run 1' was performed on a membrane preparation of high protein content, with any excess membrane protein used for 'run 2'. Four assays in 'run 2' were performed on membrane preparations of less then 200μg/ml, 3 of these had measurable receptor and the last had a very low receptor density in 'run 1'.

The results for 'run 1' and 'run 2' reveal considerable variation in measured receptor levels; results varied by a mean factor of 2.6, range 1.6 to 5.3 for each cell line. Despite this, both runs showed a degree of concordance and Spearman rank correlation analysis was statistically significant (r^2=0.604, p=0.014).

b) Radioimmunohistochemical studies

cell line	counts
BT20	1753
MDA-MB-231	901
BT474	475
SKBR3	231
MDA-MB-453	114
ZR75	104
MDA-MB-361	82
MCF7	-40
MCF7-ADR	-

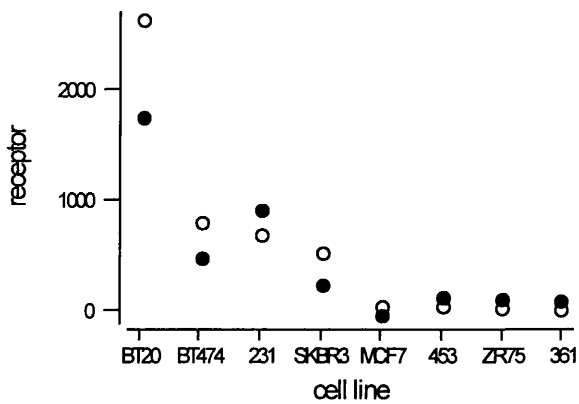
Table 6.6 : Radioimmunohistochemical results for the tumour cell lines

The counts refer to the number of silver grains counted by the image analysis system per unit area per hour of emulsion exposure, this is proportional to the number of EGF receptors present. No results were available for the MCF7-ADR line for technical reasons.

The raw data from which these results are generated is presented in Appendix 1. The negative value for the MCF7 cells indicates that counts for the control section were higher than in the test sections, this implies that, at these very low levels of receptor expression, the sensitivity of the assay was exceeded.

The figure overleaf is intended to provide a graphic impression of the relative levels of receptor expression using the different measurement methods, it should be noted that receptor is measured in distinct units for the two methods. Because the MCF7-ADR line was not studied using the radioimmunohistochemical method there are only 8 cell lines for which the results can be directly compared, Spearman rank correlation analysis, r^2=0.615, p=0.023.

Figure 6.5 : Plots of cell line receptor expression measured by ligand binding and radioimmunohistochemistry.



Solid circles represent radioimmunohistochemical results, open circles represent the results of the ligand method. Receptor levels for the ligand binding and for radioimmunohistochemistry are shown in fmol/mg membrane protein and as grains per mm^2 per hour respectively.

The results presented in this chapter are discussed in the next.

Chapter 7:
Discussion; receptor measurement

Section i: Radioimmunohistochemical measurement of EGF receptor

We have established, and validated, a radioimmunohistochemical method of measuring EGF receptor (Stanton 1994) (and c-*erb*B-2 encoded receptor - Reeves 1996). By this method EGF receptor was measurable in nearly all tumour specimens. In comparison, using conventional immunohistochemistry and ligand binding techniques receptor could not be measured in most of the tumour samples.

Using this method, it was clear that receptor expression was down-regulated in the vast majority of breast cancers (see Figure 6.1). The fact that normal breast showed detectable expression of EGF receptor has been noted in past reports (Dittadi 1993, Chrysogelos 1994). The assumption has been made that levels of expression in normal breast, whilst detectable, are low (Rajkumar 1994), and that tumours with assayable levels of EGF receptor are therefore overexpressing this marker relative to their tissue of origin. In keeping with previous reports (Dittadi 1993, Ozawa 1988) our radioimmunohistochemical results were not compatible with this. What we take to be low or high levels of receptor expression is obviously arbitrary, but within this series all but 5 tumours showed levels of expression below those of normal breast. This fitted well with the low incidence of EGF receptor gene amplification in breast cancers (Slamon 1987). However, these low levels of receptor expression may still be of functional significance as indicated by the prognostic significance of EGF receptor expression in previous studies (Sainsbury 1987, Harris 1989, Klijn 1992), and the relationship to adverse grade and oestrogen receptor status in this series. The low levels of EGF receptor detected in the tumours which we have studied was consistent with at least two explanations. It was possible that large amounts of ligand was present within the tumours, the binding of which may be resulting in internalisation of most of the receptors. That is, that the low number of cell surface receptors was a reflection of high receptor turnover (Rios 1992), rather than of low receptor production. Alternatively, there may have been low receptor production, that is true underexpression. This may have important implications for the use of this receptor as a target for tumour therapy.

It can be argued that tissue from reduction mammoplasty specimens is not the ideal control for breast cancer specimens. These patients are younger than the majority of cancer cases, and it is possible that, in normal breast lobules, receptor expression changes with age. This raises the issue of what truly normal breast tissue is available to use as a control. Histologically normal breast tissue in the cancer specimens showed the same levels of expression as our controls, however these areas may have been subject to some of the genetic changes that had resulted in the adjacent pathology. This argument is less strong for breast adjacent to benign lesions, however these also tend to occur in younger age groups. Very rarely is completely normal breast tissue removed from middle aged or older women. The other potential problem with the use of reduction mammoplasty specimens as a control is the indication for the operation itself; it is possible that breasts in these patients are very large because of overexpression of growth factor receptors. There is to our knowledge absolutely no evidence that this is so and as we have suggested areas of histologically normal breast within the tumours showed similar receptor densities. Since the consensus origin of breast carcinoma is at the level of terminal ductulo-lobular unit (Davidson 1993, Wellings 1990) lobular tissue from the reduction mammoplasty specimens was used for our control measurements.

Section ii: Comparison of measurement methods

Most reports of EGF receptor expression in breast cancer have used conventional immunohistochemical or ligand binding methods. In our series, Chi-square analysis of the results of these methods suggests a high degree of concordance (p=0.0006), however, for 13 of the tumours (26%) the results did not concur. This level of variation would account for some of the discrepancies in publications on EGF receptor.

Of the panel of 50 tumours, 38% and 48% were positive for EGF receptor using the ligand binding and conventional immunohistochemical methods respectively (Table 6.1). These rates are similar to those in the studies reviewed by Klijn (1992); 45% and 42% mean receptor positivity respectively. However, our panel of tumours was not selected in an entirely random fashion. Large tissue sections were required for ligand studies and, since all three measurement techniques were to be used, larger cancers were selected. Further, the intention of this study was to compare results of receptor measurement using the different methods, this required that a number of the tumours were receptor positive. Therefore, 10 tumours (t41 to t50) were chosen as, using radioimmunohistochemistry, they were strongly receptor positive (The investigator was unaware of this selection bias until the assays were completed.). Excluding these tumours, 23% using ligand binding and 33% using conventional immunohistochemistry were receptor positive. These results, particularly for the ligand technique, are at the lower end of the range reported in Klijn's review (1992). This may reflect small sample size, and for the ligand method a lack of familiarity with a relatively complex methodology. There are, however, other important factors that can effect studies using these methodologies.

One of these is the technique used to label the EGF ligand. The EORTC adopted EGF receptor measurement protocol (Leake 1993) advocates use of an iodine monochloride method for EGF iodination. The authors state that alternative methods oxidised the EGF molecule, causing non-equivalent binding of iodinated compared to non-iodinated ligand. This could result in a reduction in measured receptor, however, Klijn's review paper (1992) pre-dates the EORTC report and many of the reviewed studies did not use the iodine monochloride method. Our lab had extensive experience with the iodogen method but none with iodine monochloride, the former was therefore used to label ligand for our studies.

Another technical problem was that the tissues used for the ligand studies, although from the same tumour sample, were retrieved at a later date than the consecutive sections used for the immunohistochemistry. This introduced the problem of tumour heterogeneity; tumour cells within the same cancer need not express receptor uniformly, possibly reflecting different tumour cell clones (Brockhoff 1998, Beviglia 1997, Szollosi 1995). This is a problem for both ligand binding and immunohistochemistry studies. It can be argued that, since ligand studies are performed on a mass of tissue, they might more accurately reflect overall tumour EGF receptor levels. However, this has not been corroborated by better prediction of outcome in studies using ligand methodologies. Additionally, analysis of a mass of tissue introduces heterogeneity of a different type, since samples will include tissue other than breast.

Other studies may have under-estimated receptor expression by including assays performed on small tumour samples with insufficient membrane protein (Koenders 1992). Our ligand studies were performed, almost without exception (96%), on samples of more than the advised 200μg/ml membrane protein. Further, the mean membrane protein concentration in those tumours which had no measurable receptor was 856μg/ml as opposed to 688μg/ml in those in which receptor was measurable. Therefore, it was unlikely that sample weight was too small in our study.

For 8 tumours[φ] counts were higher in the control tubes, 9 and 10, (estimates of non-specific binding) than in tube 5 (see Appendix 1). Since excess cold antibody was added to control tubes this, in theory, should not have occurred. That it did may reflect pipetting error, despite efforts to minimalise this source of error (noted in table 5.2). It may also reflect sample loss, either through adherence to the eppendorf or during the repeated washing of the HAP precipitate. With greater experience of the ligand binding technique such errors might be reduced, however, that they could occur may be considered a failing of the technique itself.

Further, the ligand protocol stipulated that the counts added to the control tubes and tube 5 should be the same. A crude evaluation of the data can be made by comparing the results for these tubes. If the measured counts from tube 5 were greater than those in the control tubes, it might be expected that the tumour would have measurable receptor. Thirty tumours were identified in this manner, these included all of those identified as EGF receptor positive by the LIGAND program but also 11 others[φ]. Estimating receptor content from a single measurement point, like this, is considered to be less accurate (Leake 1993) than using a multi-point assay but several published studies have used assays with fewer than the 8 points advocated by the EORTC.

Even 8 points may be insufficient. We know this receptor exists in two affinity states, and the fact that a single site model proved superior to one with two sites indicates the imprecise nature of the ligand protocol. To achieve accurate measurement of receptor in a two binding site model, would require an assay with many more than eight points. Tumour samples would have to be much larger to maintain membrane protein content, and thereby assay sensitivity. Increased use of screening mammography means that smaller cancers are being resected, and, in future, clinicians are unlikely to jeopardise routine, but valuable, histological information to provide large amounts of tissue for EGF receptor measurement.

φ Six tumours, 2 positive and 4 negative by the LIGAND program, were excluded from these analyses as one or more of the binding data were unavailable (see Appendix 1 for raw data).

The variability of results using the ligand technique was amply demonstrated by the cell line studies. Results for the same membrane preparations, with the same protein concentrations, varied by more than 3.5 fold (Table 6.5). Yet, when the LIGAND program was used to analyse the data from both cell line 'runs', these combined results demonstrated a good accord with those from radioimmunohistochemistry (see Figure 6.5). With limited clinical material, however, repeating assays is not practical. This means that the variability seen between results for each analysis of the cell lines was likely to be reflected in the results for the tumours. For all fifty tumours, Spearman rank correlation of ligand binding and radioimmunohistochemical results revealed a high degree of correlation but this is misleading. Using ligand binding, receptor could not be measured in a significant proportion of these tumours. If these were excluded, then for the remaining tumours, there was no correlation between the techniques (Figure 6.3). Challenging the ligand technique in this fashion assumes the radioimmunohistochemical method to be accurate. This is justified by the cell line studies, where the correlation between the results of the combined analysis of ligand data and radioimmunohistochemistry was good (Figure 6.5).

In a similar comparison of the results of radio- and conventional immunohistochemistry there was a high degree of correlation (Figure 6.4). However, at lower levels of receptor expression, radioimmunohistochemistry continued to differentiate between tumours whereas the conventional method failed to do so. It is worth noting that those tumours with low or no receptor by the conventional method amounted to more than half (52%) of those studied and that these had an almost 10 fold variation in receptor expression using radioimmunohistochemistry. Again, this assumes that the radioimmunohistochemical method was accurate and that at these lower receptor levels it remained sensitive. Good evidence supports this contention. Our studies of c-*erb*B-2, in this same tumour series, have demonstrated a statistically robust inverse correlation between EGF receptor and p185c-*erb*B-2 (Robertson 1996). Although these receptors are known to interact (Earp 1995), this inverse correlation was previously unreported and was identified as a direct consequence of the accuracy of the radioimmunohistochemical method.

Conventional immunohistochemistry's lack of sensitivity is compounded by its lack of objectivity. Non-linear amplification systems are used to visualise the primary antibody and interpretation of the method product is, at least in part, subjective. In our series, all of the immunohistochemical scoring was performed by a single experienced investigator (Dr J Reeves). This may account for the very close correlation between the radio- and conventional immunohistochemistry results, but it is a luxury not universally available. Other authors have alluded to the difficulty of satisfactorily grading immunohistochemical staining (Sallinen 1994, van Diest 1996). Clearly this could effect the reproducibility of study results, a problem that also afflicts the ligand method. Leake (1993), using the EORTC ligand protocol, reported considerable inter-laboratory variation and suggested extended quality control was imperative. Inter-observer variation is further complicated by tumour heterogeneity. Both problems can be reduced by computer assisted scoring systems (Sullinen 1994). These obviate tedious scoring and can reduce the influence of heterogeneity by analysing large areas of tumour. With computer assisted scoring and standard sections, radioimmunohistochemistry could facilitate multi-centre studies.

Overall, radioimmunohistochemistry had a number of advantages over conventional methods. It was more sensitive, both conventional methods failed to detect receptor in most of the tumour samples. Also, it was more reproducible. Finally, the use of computer assisted scoring made it more accurate.

Did these improvements translate into clinical advantage? This question is addressed in the subsequent chapters.

Chapter 8:
EGF receptor; relation to clinical variables and outcome

Section i: Database construction

Data for the 203 tumours studied using immunohistochemistry were collected. These tumours were all surgical treated at Glasgow Royal Infirmary between 1984 and 1993. Pathological details were recorded by the pathologist reporting on that specimen. Tumour size was taken as the largest diameter, determined macroscopically, and was recorded in millimetres. The TNM classification was used to categorise tumours for statistical analysis. The T4 category, extension to chest wall or skin, could not be reliably determined from the case records or pathology reports and was not used. Numbers of nodal metastases and nodes sampled were taken from the routine pathology reports. During the period over which the tumours were collected there was a shift in practice from node sampling toward axillary clearance and this was reflected in the numbers of nodes retrieved. In the early part of the study period, oestrogen receptors were assayed in frozen tumour samples using the dextran coated charcoal method with a cut off of 20 fmol/mg. Later this method was replaced by an immunohistochemical technique. Using this tumours were scored from 0-300 in a analysis similar to that described for conventional immunohistochemical measurement of EGF receptor (Chapter 5, section iv). Data from both methods were used in the analyses. Modified Bloom and Richardson grade (Elston 1991) was recorded by the pathologist reporting the specimen. Prior to 1992 this parameter was not routinely measured and the grade of these tumours was assessed retrospectively by one pathologist (Dr James Going).

Follow-up data were obtained from the patients' medical records. Glasgow Royal Infirmary breast cancer patients are reviewed at a dedicated clinic and in keeping with this these patients have breast clinic case records as well as general records. Both sets of records were used to provide comprehensive follow-up information. Clinical correspondence was copied to the investigators who also had access to the case records. In this manner the database was kept up to date. Collated information included end points; local recurrence, distal recurrence and death, and adjuvant therapy; tamoxifen, radiotherapy and chemotherapy. Death was not always available from the case records and was confirmed using the cancer registry at the Cancer Surveillance Unit at Ruchill hospital, Glasgow (Dr D Hole). From the International Classification of Diseases (ICD) codes it was possible to determine if death was breast cancer related or otherwise. Scottish Cancer

Registry data were recognised to be of high quality, serious discrepancies occurring in as few as 2.8% of records (Brewster 1994).

Data were stored in a relational database using the Microsoft Access program (Version 2.00, Copyright © 1989-1994 Microsoft Corporation, USA). The database was established in 1994 and, for the purposes of this report, was closed on 1st January 1998. The relationship between EGF receptor expression and other prognostic factors was studied using the Minitab for Windows software (Release 9.2, Copyright © 1993, Minitab Inc.). The life table analyses were performed using the SPSS program (SPSS for Windows, Release 6.1.3, Copyright © SPSS Inc. 1989-95). Appendix 2 contains the raw data for the studies presented in this chapter.

Section ii: Results - prognostic factors

Of the 203 tumours studied, nine were excluded from subsequent analyses. Eight of these because the patients had had a previous breast cancer resected and one because the tumour was thought to be a metastasis from an oesophageal primary. Mean age of the patients was 61 years (range 28 to 87 years). Median EGF receptor density was 5% of levels found in normal breast (range 0 to 707%, see Figure 6.1). The relationships between receptor expression and prognostic factors are summarised in table 8.1.

Tumour size was available for all but 5 tumours. Median tumour size was 25mm (range, 6 to 130). Using the TNM classification there were 44, 121 and 24 tumours of T1, T2 and T3 stages respectively. There was no significant difference in the median level of EGF receptor in the three T-stage groups, however, tumour size, in millimetres, correlated directly to EGF receptor expression (Spearman rank correlation analysis, $p=0.049$, $n=188$, $r^2=0.021$). This relationship is plotted in figure 8.1.

Modified Bloom and Richardson grade was available for all but one cancer. There were 25 (13%), 73 (38%) and 95 (49%) tumours of grade 1, 2 and 3 respectively. Tumours classified as grade 2 expressed lower levels of receptor than did those of grade 1, but this difference did not achieve statistical significance (Mann Whitney *U* test, $p=0.723$). However, grade 3 tumours expressed much more receptor than those of either grade 1 or 2, and this finding was statistically significant (Table 8.1). The lack of an increment in receptor expression with each grade may reflect the small number of tumours categorised as grade 1. Overall, higher grade, more poorly differentiated cancers tended to express the highest levels of EGF receptor.

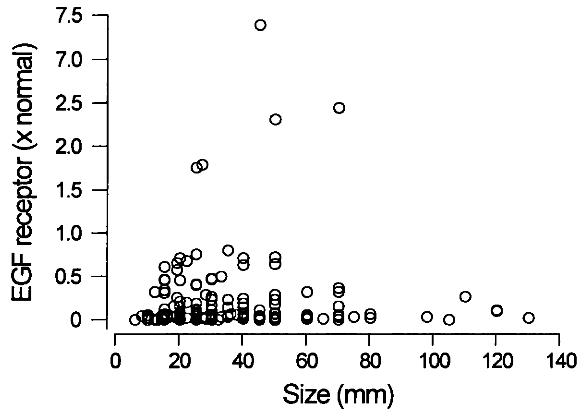


Figure 8.1: Plot of EGF receptor (radioimmunohistochemical measurement) and tumour size (millimetres). Spearman rank analysis p=0.049, n=188, r^2=0.021.

Table 8.1: Relationship between prognostic factors and EGF receptor expression.

		n (%)	medianEGFr (x normal)	
Tumour size	T1	44 (23)	0.041	
(n=189)	T2	121 (64)	0.048	
	T3	24 (13)	0.045	p=0.392
Hist. Grade	GI	25 (13)	0.047	
(n=193)	GII	73 (38)	0.035	
	GIII	95 (49)	0.078	p=0.005
ER status	neg	61 (44)	0.194	
(n=140)	pos	79 (56)	0.032	p<0.0005
Nodal status	neg	83 (49)	0.043	
(n=170)	pos	87 (51)	0.056	p=0.074

For tumour size and Histological grade Kruskall-Wallis analysis was used, for Nodal status and ER status Mann-Whitney *U* test was employed. The TNM classification was used for tumour size and Modified Bloom and Richardson score (Elston 1991) for histological (Hist.) grade. ER and EGFr indicate oestrogen and EGF receptors respectively.

As indicated above oestrogen receptor status was determined using two different methods. In total, it was available for 151 (78%) of the tumours. Of these, seventy were studied using both techniques. All tumours that were oestrogen receptor positive using the dextran coated charcoal method were positive using immunohistochemistry. Three tumours were receptor negative by the former technique but were positive by the latter, for purposes of statistical analysis these were considered receptor positive. With receptor expression categorised as positive or negative, there was a strong indirect relationship between EGF and oestrogen receptor.

To further analyse this relationship, measured receptor levels, for the oestrogen receptor positive tumours, were compared. For the ligand method there was no correlation (Spearman rank correlation analysis, p=0.282, n=74, r^2=0.002), but results of the immunohistochemical method correlated indirectly with EGF receptor levels (Spearman rank analysis, p=0.032, n=53, r^2=0.069).

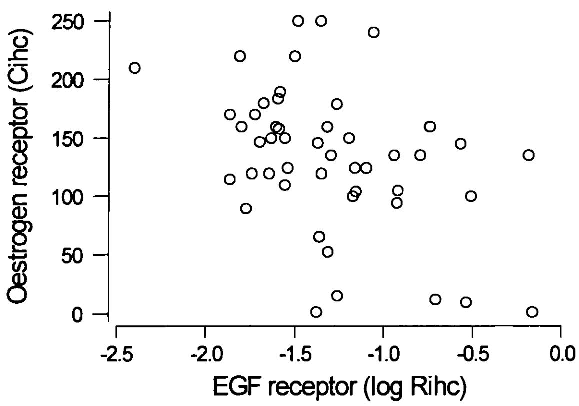


Figure 8.2: Plot of oestrogen receptor levels (measured using conventional immunohistochemistry) and EGF receptor levels (shown as $\log_{10}$ of radioimmunohistochemistry (Rihc)). Spearman rank analysis p=0.032, n=53, r^2=0.069.

Information on node sampling was available on 186 (96%) of the tumours. In 16 of these no nodal tissue was found by the pathologist. The mean number of nodes retrieved was 5.9 (range, 0 to 24 nodes). Therefore nodal information was available for 170 of the cancers, of which 87 (51%) were node positive. In 31 tumours (18%) more than three nodes were involved. For those tumours with available nodal information, node involvement bore no statistically significant relationship to EGF receptor expression levels.

For any given tumour the number of sampled nodes varied widely and a sub-analysis of tumours with 4 or more sampled nodes was performed. Tumours were categorised as node negative, 1 to 3 nodes involved and more than 3 involved nodes, these groupings define cohorts with distinct prognoses; 10 year survival of 64.9%, 37.5% and 13.4% respectively (Miller 1994). Two analyses were performed. In the first, tumours were categorised as node negative or node positive, as above. In the second, the node positive group were split into those with fewer than 4 involved nodes and those with 4 or more involved nodes. The table below shows the results

Table 8.2: Subanalysis of nodal status

		n (%)	median EGFr (x normal)	
Nodal status	neg	63 (49)	0.047	
(n=128)	pos	65 (51)	0.044	p=0.576
Nodal groups	0	63 (49)	0.047	
(n=128)	1-3	34 (27)	0.052	
	>3	31 (24)	0.041	p=0.680

Analysis was limited to those cases in which 4 or more nodes were sampled. In the analysis of nodal status cancers were coded as node negative or node positive. Statistical analysis was by Mann-Whitney *U* test. In the second analysis, tumours were split into nodal groups, no nodes involved, 1-3 nodes involved and more than 3 nodes involved. A Kruskall-Wallis statistical analysis was applied to this study. EGFr indicates EGF receptor.

This sub-analysis confirmed that there was no statistically significant relationship between EGF receptor expression and nodal status.

Section iii: Results - adjuvant therapy

Adjuvant systemic therapy, tamoxifen or chemotherapy, was prescribed for 148 (76%) patients. Only 13 (7%) patients had no adjuvant systemic therapy (data unavailable for 33). 130 patients received adjuvant tamoxifen therapy and 35 did not (data unavailable for 29). Chemotherapy was prescribed for 26 but not for 138 (data unavailable for 30). Only one patient received both. The mean age of those receiving tamoxifen was 64 years (range, 42-87) and those receiving chemotherapy was 47 years (range, 29-68). Radiotherapy was prescribed for

61 patients (31%), 102 patients (53%) did not have this treatment whilst for 31 the case records failed to make it clear if radiotherapy had, or had not, been given.

No further analysis of adjuvant therapy data was made for reasons given in Chapter 9.

Section iv: Results - outcome

Follow-up commenced at the date of histological diagnosis or surgery. End points were local recurrence, distal recurrence and death. For purposes of analysis data collection continued until the end of 1997. Data were available for all but 3 patients. Case sheets for these patients could not be traced, but none was recorded as dead at the cancer registry. They were therefore assumed to be alive. At the end of 1997, 91 patients (47%) were alive. The mean duration of follow-up for this cohort, excluding the 3 cases with no follow-up, was 2282days (range, 162 to 4445). In total, 103 patients (53%), with a mean follow-up of 1145days, had died. These patients could be dichotomised using the cancer deaths registry data to those who had breast cancer related deaths and those who did not. 81 patients (42%) died from causes directly attributed to breast cancer, mean follow-up 1120days (range, 89 to 2804). Those whose death certificates did not register breast cancer numbered 22 (11%), mean follow-up 1236days (range 21 to 3138). Survival analyses were performed on groups defined by both breast cancer related deaths (Disease specific survival) and all deaths (Overall survival).

Many publications measure outcome in terms of recurrence (i.e. disease free survival) as well as death. To monitor disease recurrence, the breast cancer clinic at Glasgow Royal Infirmary had a surveillance policy. All patients had mammography at 3 year intervals, those who had had local resections underwent annual mammography for 5 years. Ideally, all patients had annual chest and pelvic x-rays as well as liver function tests. However, the enthusiasm with which this protocol was applied varied with the reviewing clinician. Use of other diagnostic tools was prompted chiefly by clinical suspicion. Most frequently, these were fine needle aspiration biopsies of suspected local recurrence, and for distal disease, bone scans, liver ultrasonography and brain CT scans.

For the purposes of this study, local recurrence was recorded if histologically proven or if there was a high index of clinical suspicion, most often resulting in a modification of therapy. Distal recurrence was rarely histologically determined and was based chiefly on results of symptom prompted diagnostic imaging.

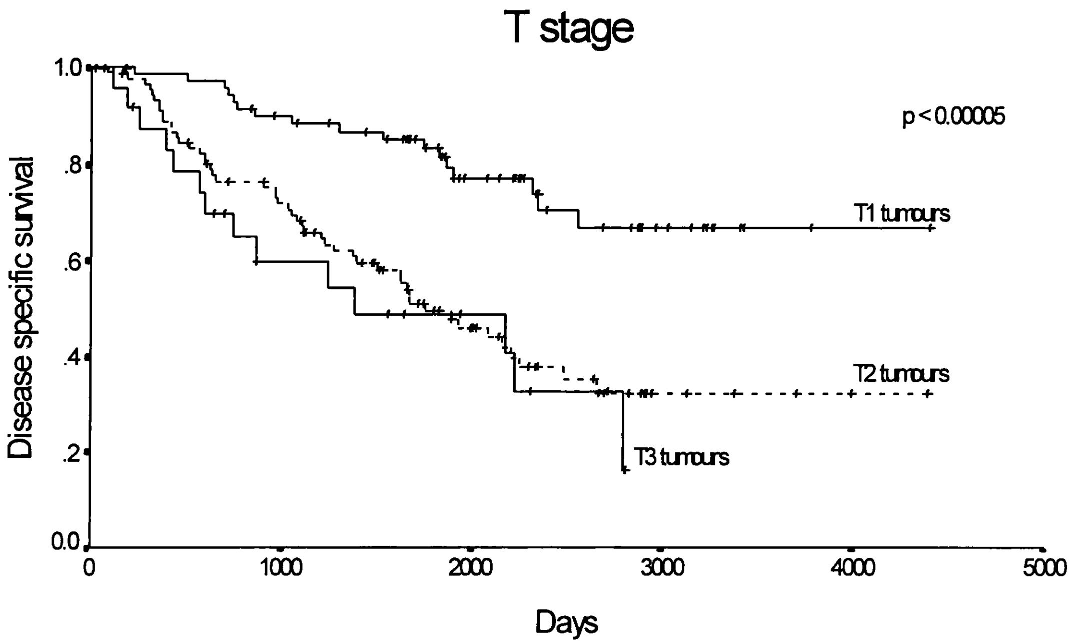
In subsequent analyses, those patients with breast cancer related deaths were considered to have recurred at a distant site. Those alive, at the termination of the study, in whom recurrent disease was not diagnosed, were considered to be free of recurrence. Recurrence of any kind occurred in 99 patients (51%) at a mean duration of 767days. Distal recurrences occurred in 90 patients (46%) at a mean duration of 884days (range 0 to 2485) and local recurrences in 48 patients (25%) at 806days (range 43 to 3235).

On the following pages are the results of both univariate (Kaplain-Meier) and multivariate (Cox regression) analyses of the follow-up database. Patients were divided into groups of equal number using the radioimmunohistochemical measure of EGF receptor content of their tumours; 2 groups of 97 cases (cut-off 0.047 times normal breast levels), 3 groups, 65, 65 and 64 cases (cut-offs 0.030 and 0.079 times normal breast levels) and 4 groups, 48, 49, 48 and 49 cases (cut-offs 0.022, 0.047 and 0.158 times normal breast levels). Both statistics and graphics were generated using the SPSS program. Appendix 2 contains the raw data for these studies.

a) Univariate analyses

The aims of this thesis relate to the EGF receptor, therefore most of the analyses concern this variable. However, to characterise the database analyses of tumour size, oestrogen receptor content, histological grade and nodal involvement were performed.

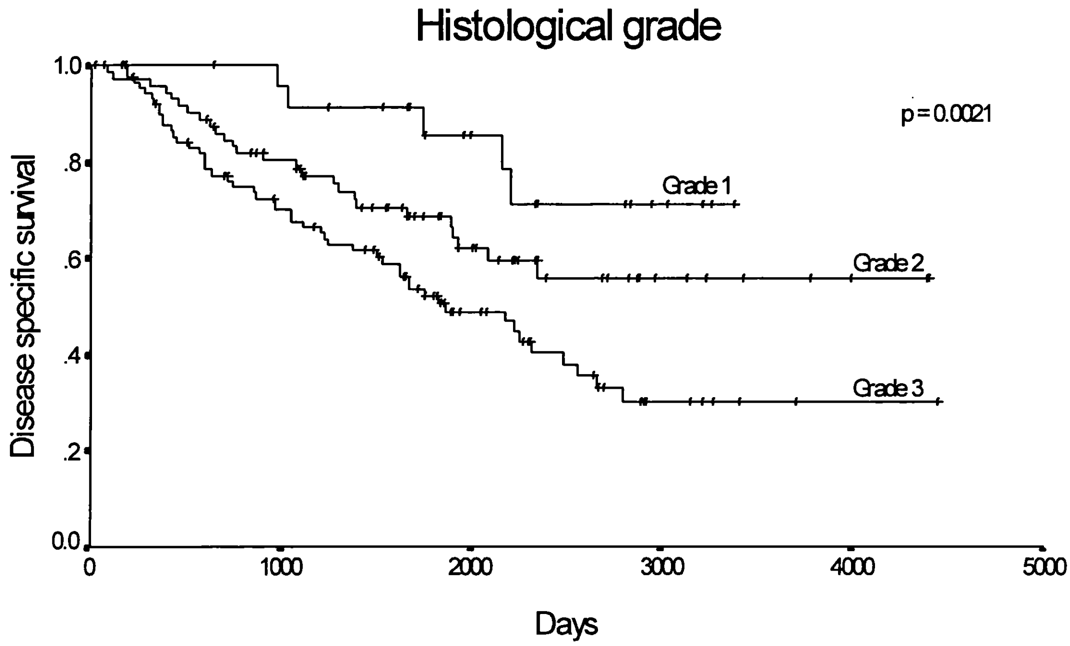
Figure 8.3 : Kaplan-Meier analysis of the relationship between T stage and survival.



Disease specific survival is indicated on the y-axis, the scale shows the proportion of patients remaining alive at the follow-up time point indicated, in days, on the x-axis. Tumours were classified into three groups, one to three, of progressively larger size using the TNM classification (no tumours were categorised as T4).

Tumour T stage (TNM) strongly predicted for death from breast cancer related causes (Kaplan-Meier statistic 22.15, d.f. 2, $p<0.00005$). It remained a strong predictor of survival when all deaths were considered (Kaplan-Meier statistic 26.63, d.f. 2, $p<0.00005$).

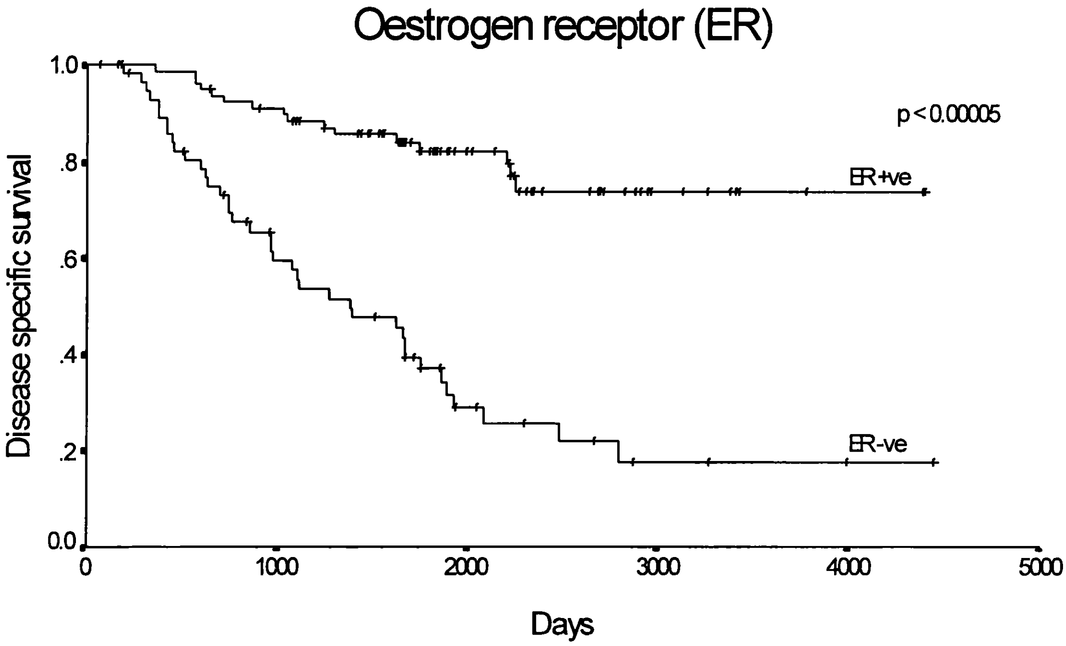
Figure 8.4 : Kaplan-Meier analysis of the relationship between histological grade and survival.



Disease specific survival is indicated on the y-axis, the scale shows the proportion of patients remaining alive at the follow-up time point indicated, in days, on the x-axis. Tumours were classified into three groups, one to three, of progressively poorer differentiation using a modified Bloom and Richardson grading system (Elston 1991).

Histological grade also proved a good predictor of outcome in terms of breast cancer related deaths (Kaplan-Meier statistic 12.30, d.f. 2, p=0.0021) and deaths from all causes (Kaplan-Meier statistic 9.86, d.f. 2, p=0.0072).

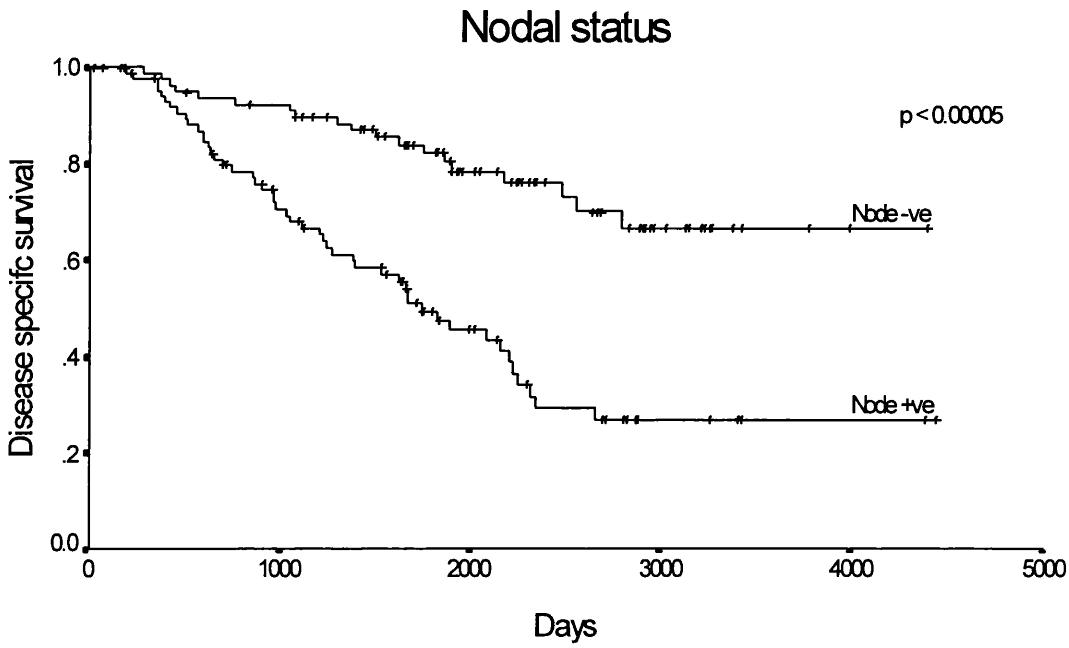
Figure 8.5 : Kaplan-Meier analysis of the relationship between oetrogen receptor status and survival.



Disease specific survival is indicated on the y-axis, the scale shows the proportion of patients remaining alive at the follow-up time point indicated, in days, on the x-axis. Oestrogen receptor status was determined as described above. ER +ve and ER -ve indicate those tumours identified as oestrogen receptor positive and negative respectively.

Oestrogen receptor status was an exceptionally strong predictor of death from breast cancer related causes (Kaplan-Meier statistic 37.77, d.f. 1, $p<0.00005$). It remained so when all deaths were considered (Kaplan-Meier statistic 34.90, d.f. 1, $p<0.00005$).

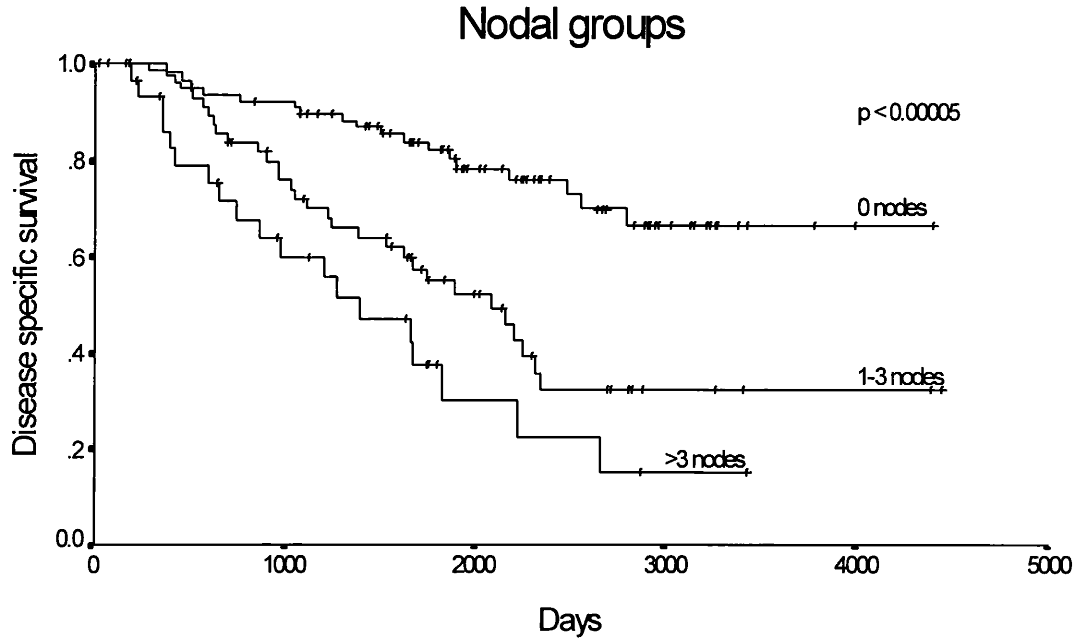
Figure 8.6 : Kaplan-Meier analysis of the relationship between nodal status and survival.



Disease specific survival is indicated on the y-axis, the scale shows the proportion of patients remaining alive at the follow-up time point indicated, in days, on the x-axis. Tumours were classified as having histologically confirmed nodal metastases, node +ve, or as being free of nodal metastases, node -ve.

Presence of nodal metastases predicted breast cancer related mortality (Kaplan-Meier statistic 23.39, d.f. 1, $p<0.00005$). It also predicted overall survival (Kaplan-Meier statistic 18.76, d.f. 1, $p<0.00005$).

Figure 8.7 : Kaplan-Meier analysis of the relationship between nodal groups and survival.



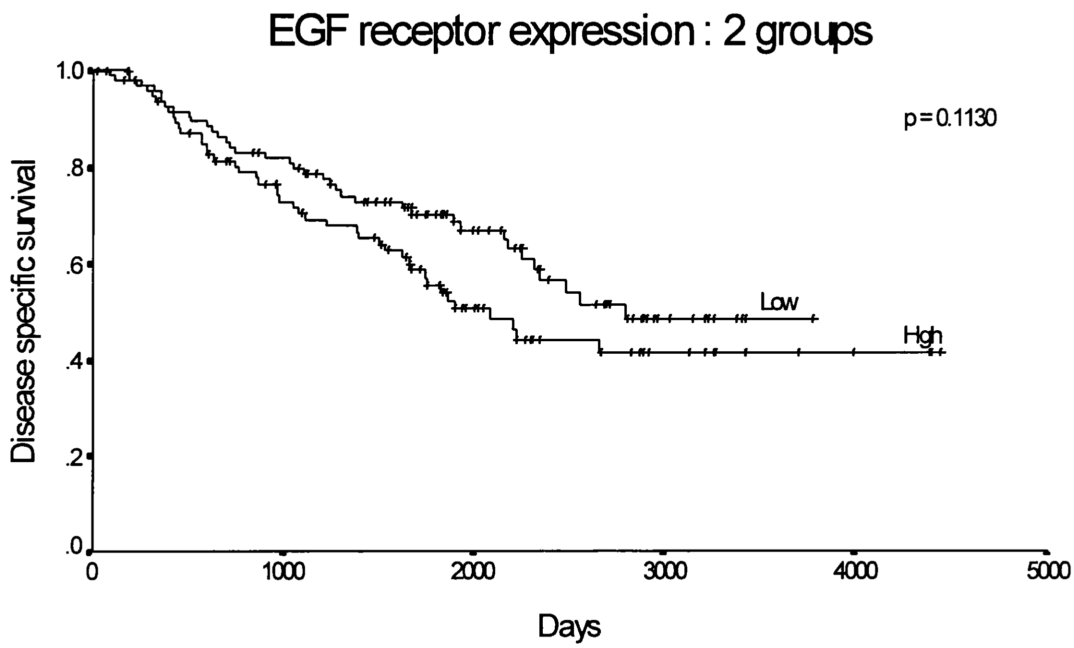
Disease specific survival is indicated on the y-axis, the scale shows the proportion of patients remaining alive at the follow-up time point indicated, in days, on the x-axis. Tumours were classified into three groups determined by the number of histologically determined nodal metastases; no nodal metastases (0 nodes), one to three involved nodes (1-3 nodes) and more than three nodal metastases (>3 nodes).

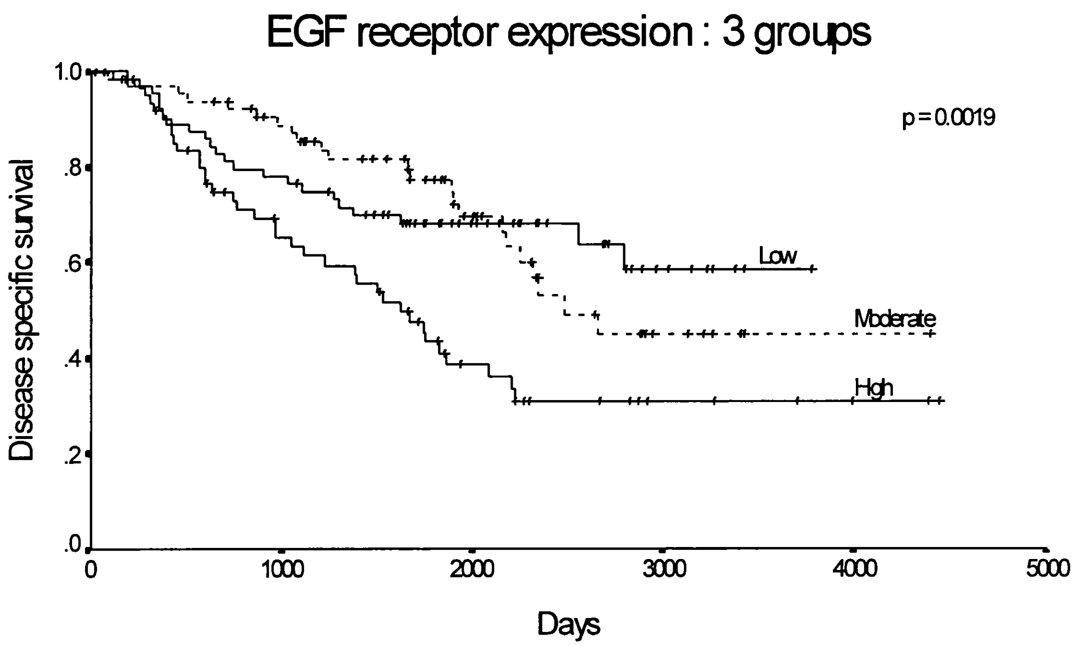
Analysing the tumours depending on the number of involved nodes also predicted breast cancer related deaths (Kaplan-Meier statistic 28.58, d.f. 2, $p<0.00005$). For overall survival these groupings also proved predictive (Kaplan-Meier statistic 28.45, d.f. 2, $p<0.00005$).

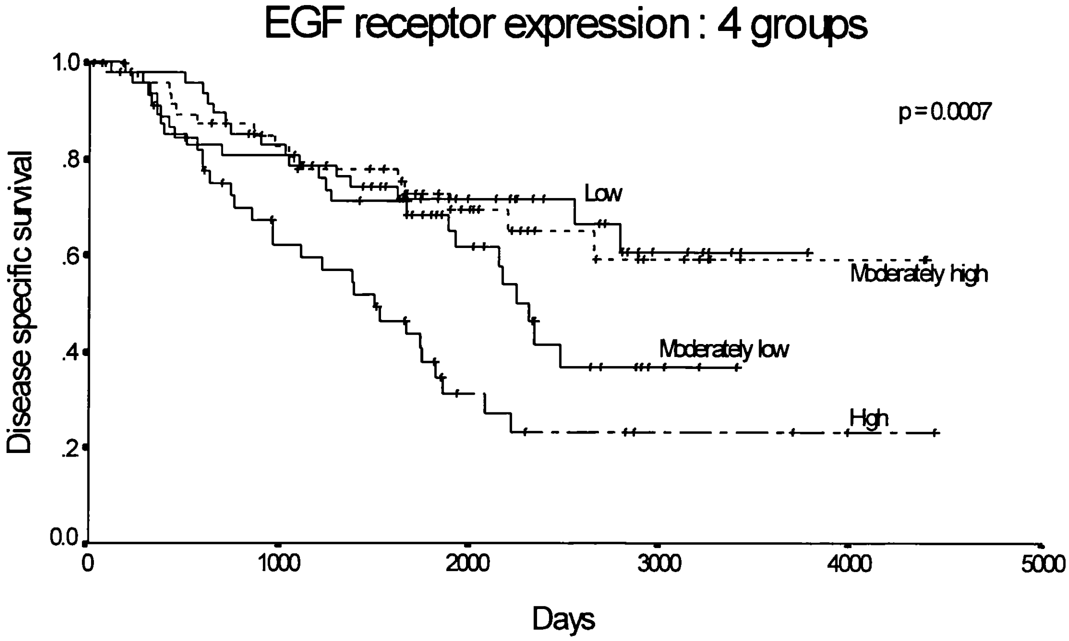
The unusually strong predictive power of oestrogen receptor status in this series is discussed in chapter 9. The results for the remaining conventional prognostic factors reflect those reported in the literature. Subsequent univariate analyses explored the relationship between EGF receptor expression and outcome.

Survival Analysis - Disease specific and Overall survival

Figure 8.8 : Kaplan-Meier analyses of the relationship between survival and EGF receptor. Patients were split into; 2 groups, 3 groups and 4 groups, using the levels of EGF receptor expression in their tumours.







Disease specific survival is indicated on the y-axis, the scale shows the proportion of patients remaining alive at the follow-up time point indicated, in days, on the x-axis. Groups are categorised, in ascending order of expression, as; low, moderately low, moderately high and high. Plot annotations refer to the line to which they are closest. Dotted and dashed lines have been used where there is overlap to try and minimise confusion.

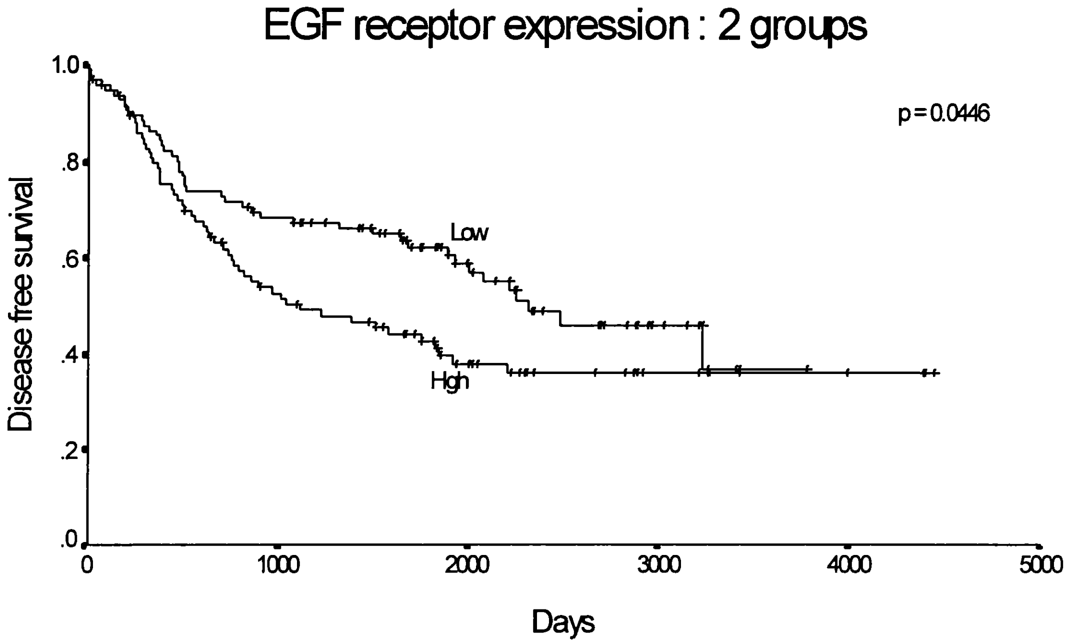
The Kaplan-Meier analyses are summarised below.

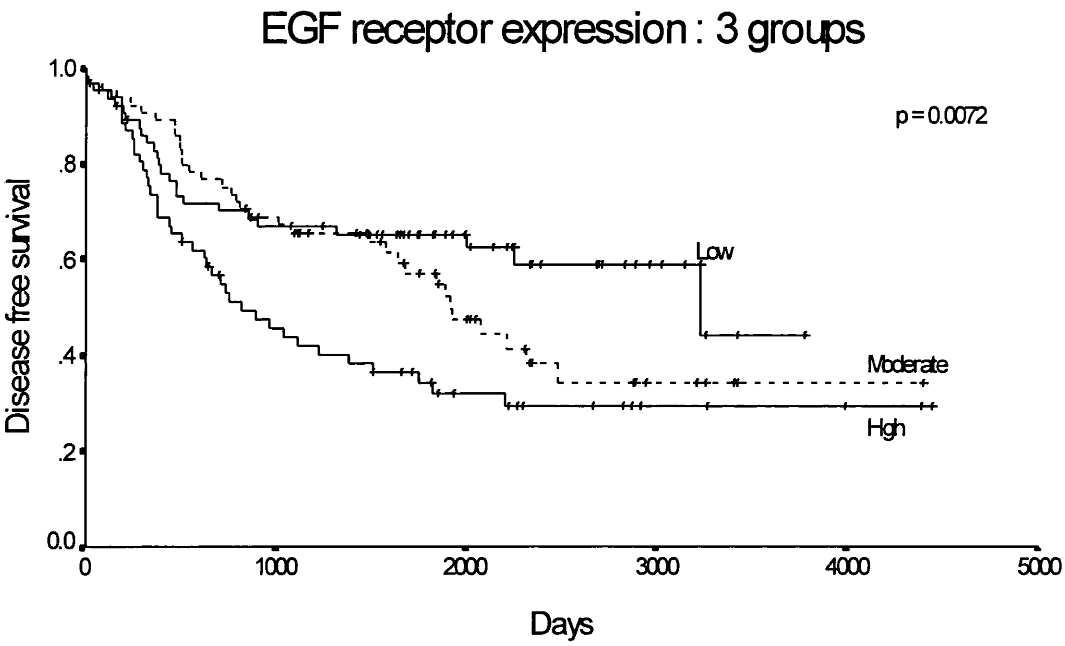
	Kaplan-Meier statistic	d.f.	p value
2 groups / disease specific survival	2.51	1	0.1130
2 groups / overall survival	4.10	1	0.0429
3 groups / disease specific survival	12.50	2	0.0019
3 groups / overall survival	10.70	2	0.0047
4 groups / disease specific survival	17.14	3	0.0007
4 groups / overall survival	16.48	3	0.0009

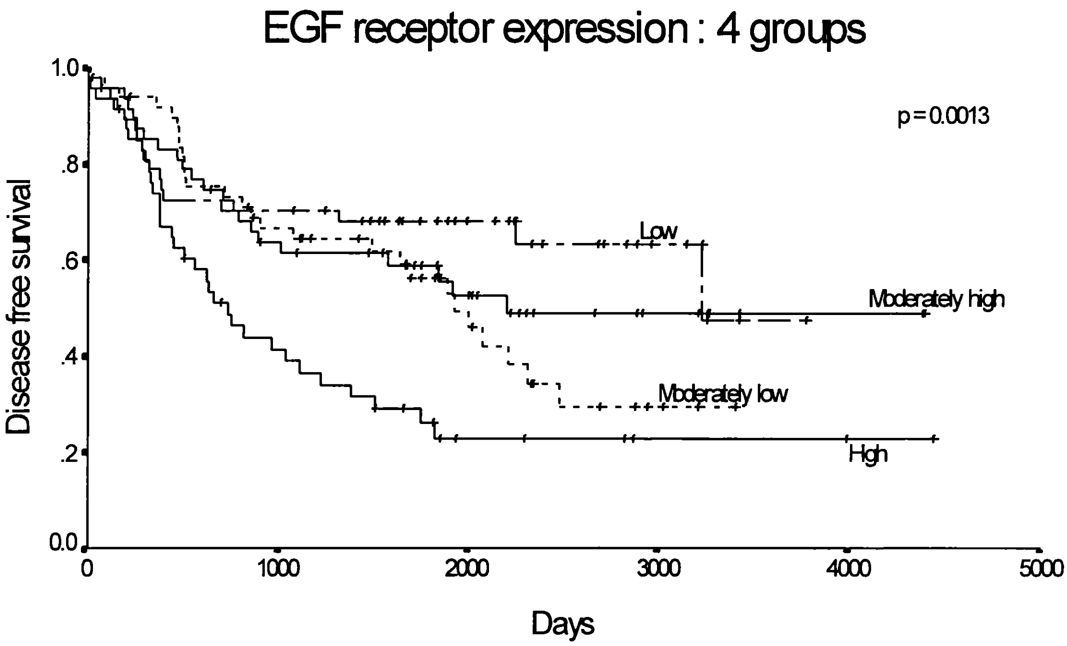
For disease specific survival, splitting EGF receptor expression into two groups did not produce a statistically significant separation of survival curves. For overall survival this dichotomy was just significant. When receptor expression was split into 3 groups these had a statistically different outcome. For 4 groups the statistical power seemed greater but the survival curves for the moderately high and moderately low cohorts overlapped so that the relationship between outcome and receptor expression was not incremental.

Survival Analysis - Disease free survival

Figure 8.9 : Kaplan-Meier analyses of the relationship between disease free survival and EGF receptor.







Patients were split into; 2 groups, 3 groups and 4 groups, using the levels of EGF receptor expression in their tumours. Axes and figure annotations are similar to those in Figure 8.8

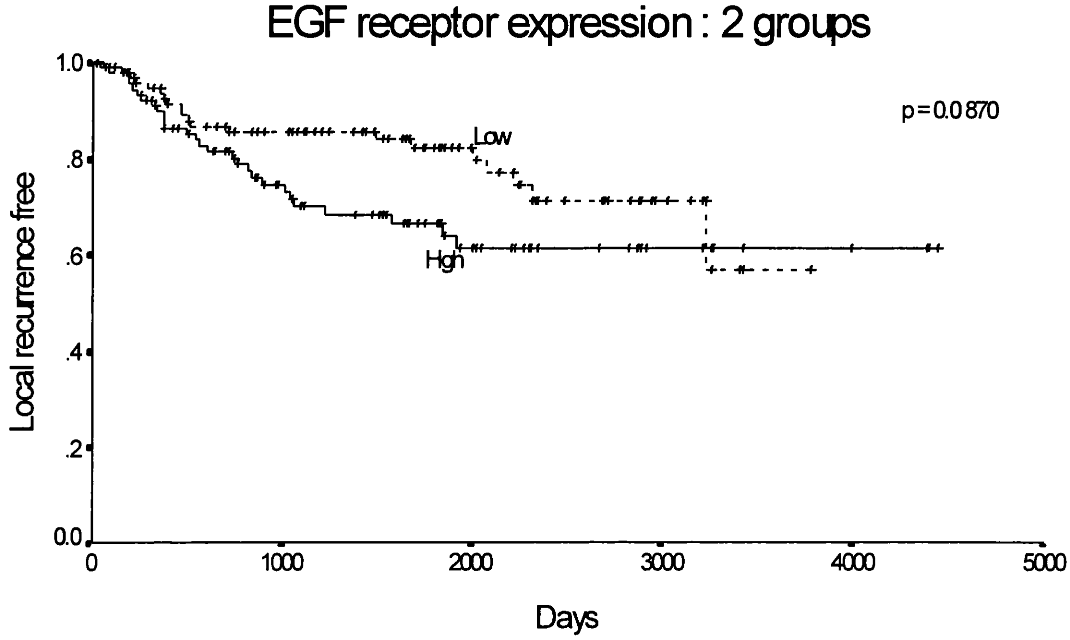
The Kaplan-Meier analyses are summarised below.

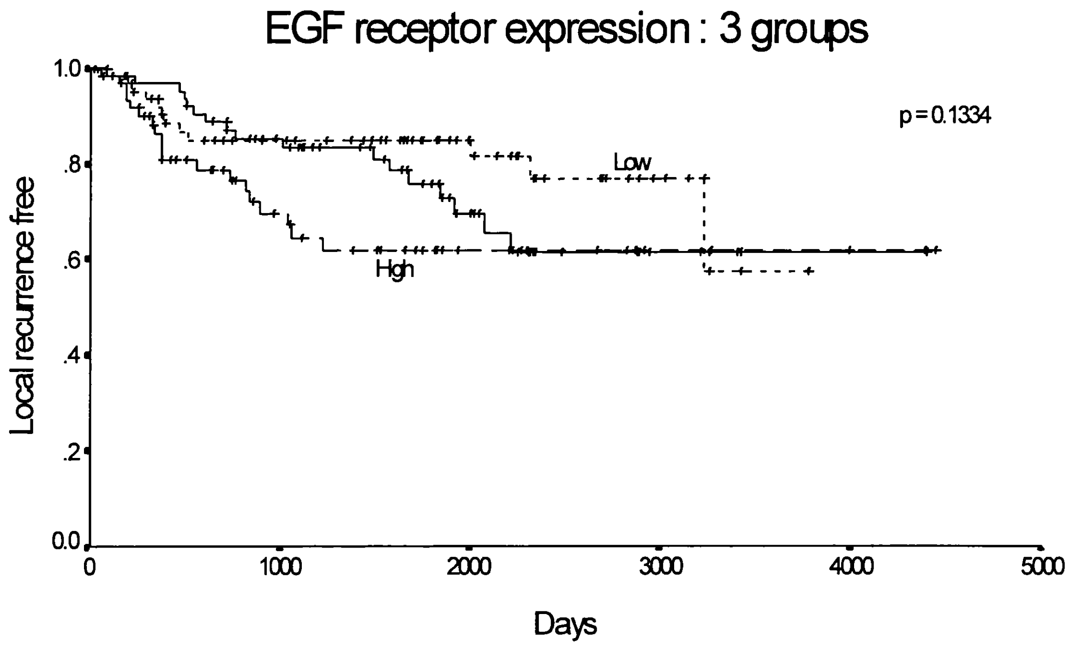
EGF receptor expression	Kaplan-Meier statistic	d.f.	p value
2 groups	4.03	1	0.0446
3 groups	9.88	2	0.0072
4 groups	15.79	3	0.0013

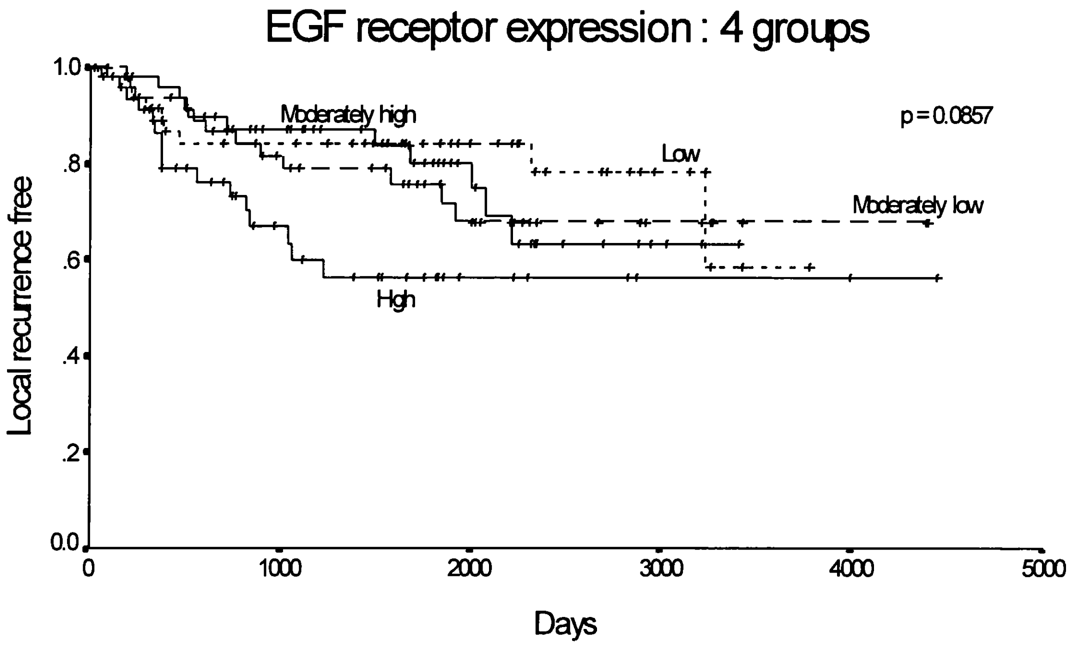
The statistical power of these analyses increased with the number of groups into which tumours were divided. As before, there was some overlap of the survival curves and this was most prominent when the tumours were split into 4 groups. In the analysis of 3 groups the low and moderate expression curves follow a similar course until approximately 5 years of follow-up, they then begin to diverge.

Survival analysis - Local recurrence

Figure 8.10 : Kaplan-Meier analyses of the relationship between local recurrence and EGF receptor. Patients were split into; 2 groups, 3 groups and 4 groups, using the levels of EGF receptor expression in their tumours. Axes and figure annotations are the similar to those in Figure 8.8







The Kaplan-Meier analyses are summarised below.

EGF receptor expression	Kaplan-Meier statistic	d.f.	p value
2 groups	2.93	1	0.870
3 groups	4.03	2	0.1334
4 groups	6.60	3	0.0857

None of these analyses was statistically significant. However, the curves themselves tended to suggest that local recurrence was more frequent in those tumours with higher receptor expression.

b) Multivariate analyses

Cox regression, performed in a forward stepwise conditional manner, was used for all analyses. The variables included in these studies were; T stage, nodal status, oestrogen receptor status, histological grade and EGF receptor expression. The last was analysed in 2 groups (EGFr-2), 3 groups (EGFr-3), 4 groups (EGFr-4), and as a continuous variable (as a factor of levels in normal breast, EGFr-c).

Note that, for all these analyses, relative risk is expressed as a factorial increase in risk for each unit increment of the appropriate variable. For nodal status this increment was a change from node negative to node positive (N0 to N1), for oestrogen receptor, it was a change from oestrogen receptor positive to negative.

An increase of one TNM stage, and one modified Bloom and Richardson grade, was used for T-stage, and Grade, respectively. When EGF receptor expression was analysed in groups risk was expressed for each increment of one group. When it was studied as a continuous variable, the risk increment was one times the mean level of receptor in normal breast (as measured in Chapter 6).

Analysis of all factors

Table 8.3 : Cox regression analysis of disease specific survival - analysis of all prognostic factors

Variables in the equation	p value	Relative risk	95% CI
ER status	<0.00005	4.7	2.5 - 8.7
Node status	0.0009	3.0	1.6 - 5.7
T stage	0.0158	1.8	1.1 - 2.8
Variables *not* in the equation	p value		
EGFR-2	0.7421	-	-
EGFR-3	0.7954	-	-
EGFR-4	0.7262	-	-
EGFR-c	0.4784	-	-
Grade	0.3766	-	-

For tables 8.3 and 8.4, 123 cases with 49 breast cancer related deaths and 126 cases with 60 recurrences, were analysed, respectively. In both tables, the relative risk is the increased risk for each unit increase in that variable. EGF receptor expression levels were used to split the patients into groups of equal size; 2 groups (EGFR-2), 3 groups (EGFR-3) and 4 groups (EGFR-4). EGF receptor levels were also studied as a continuous variable (EGFR-c). The other variables are defined at the beginning of this section. The 95% CI is the 95% confidence interval for the relative risk. Relative risk, and its confidence limits, are given only for those variables included in the equation (i.e. predictive of outcome).

Table 8.4: Cox regression analysis of disease free survival - all prognostic factors

Variables in the equation	p value	Relative risk	95% CI
ER status	<0.00005	3.1	1.8 - 5.2
Node status	0.0041	2.3	1.3 - 4.0
T stage	0.0047	1.8	1.20 - 2.7
Variables *not* in the equation	p value		
EGFR-2	0.6812	-	-
EGFR-3	0.7436	-	-
EGFR-4	0.6744	-	-
EGFR-c	0.7109	-	-
Grade	0.0603	-	-

The key to Table 8.4 is given below Table 8.3

Analysis of overall survival produced similar results. 126 cases were studied with 58 deaths. The same prognostic factors were predictive: ER status, $p<0.00005$, relative risk 4.3, 95% CI, 2.4 - 7.5; Node status, $p=0.0047$, relative risk 2.3, 95% CI, 1.3 - 4.0; T stage, $p=0.0060$, relative risk 1.8, 95% CI, 1.2 - 2.7.

Subgroup analysis - node status

In most series, presence of nodal metastases is the prognostic feature with greatest predictive power and may influence adjuvant therapy decisions. To ascertain if EGF receptor expression might determine prognostic subgroups within patient populations determined by lymph node status the survival analyses were repeated in patient groups defined by nodal status. (Tables 8.5 and 8.6)

Table 8.5: Cox regression analysis of disease specific survival - node status

Lymph node negative tumours (59 cases with 13 events)

Variable	ER status	T stage	Grade	EGFr-2	EGFr-3	EGFr-4	EGFr-c
p-value	0.0006	0.4260	0.5923	0.7635	0.8590	0.8858	0.1731
relative risk	14.0	-	-	-	-	-	-
95% CI	3.1-63.3	-	-	-	-	-	-

Lymph node positive tumours (64 cases with 36 events)

Variable	ER status	T stage	Grade	EGFr-2	EGFr-3	EGFr-4	EGFr-c
p-value	0.0004	0.0533	0.4226	0.9925	0.9897	0.9527	0.2642
relative risk	3.5	-	-	-	-	-	-
95% CI	1.7-7.0	-	-	-	-	-	-

Table 8.6: Cox regression analysis of disease free survival - node status

Lymph node negative tumours (59 cases with 18 events)

Variable	ER status	T stage	Grade	EGFr-2	EGFr-3	EGFr-4	EGFr-c
p-value	0.0348	0.0290	0.3273	0.8696	0.8176	0.8785	0.3793
relative risk	2.9	2.3	-	-	-	-	-
95% CI	1.1-7.9	1.1-5.0	-	-	-	-	-

Lymph node positive tumours (65 cases with 42 events)

Variable	ER status	T stage	Grade	EGFr-2	EGFr-3	EGFr-4	EGFr-c
p-value	0.0017	0.0761	0.0963	0.6365	0.5668	0.4728	0.4135
relative risk	2.8	-	-	-	-	-	-
95% CI	1.5-5.2	-	-	-	-	-	-

In both tables, relative risk is the increased risk for each unit increase in that variable (defined in the legend of page 92). Relative risk, and its confidence limits, are given only for those variables predictive of outcome.

Analysis of overall survival showed that ER status was the only predictor of outcome: in node negative disease; p=0.0004, relative risk 5.7, 95% CI, 2.2-15.0, and in node positive disease; p=0.0001; relative risk 3.8, 95% CI, 1.9-7.5

Subgroup analysis - oestrogen receptor status

The predictive value of EGF receptor expression might vary within groups defined by oestrogen receptor status. This possibility was explored by repeating the survival analyses for cohorts with oestrogen receptor positive and negative tumours respectively. Results are shown in tables 8.7 and 8.8.

Table 8.7: Cox regression analysis of disease specific survival - oestrogen receptor status

Oestrogen receptor positive tumours (73 cases with 15 events)

Variable	nodal status	T stage	Grade	EGFr-2	EGFr-3	EGFr-4	EGFr-c
p-value	0.0049	0.1451	0.2001	0.7146	0.8871	0.6078	0.0015
relative risk	8.5	-	-	-	-	-	72.8
95% CI	1.9-38.1	-	-	-	-	-	5.1-1030.1

Oestrogen receptor negative tumours (50 cases with 34 events)

Variable	nodal status	T stage	Grade	EGFr-2	EGFr-3	EGFr-4	EGFr-c
p-value	0.0483	0.1706	0.8625	0.5716	0.2363	0.4400	0.7286
relative risk	2.1	-	-	-	-	-	-
95% CI	1.0-4.4	-	-	-	-	-	-

Table 8.8: Cox regression analysis of disease free survival - oestrogen receptor status

Oestrogen receptor positive tumours (73 cases with 23 events)

Variable	nodal status	T stage	Grade	EGFr-2	EGFr-3	EGFr-4	EGFr-c
p-value	0.1744	0.0004	0.0809	0.6983	0.3605	0.5317	0.0017
relative risk	-	2.8	-	-	-	-	39.0
95% CI	-	1.6-4.9	-	-	-	-	4.0-384.3

Oestrogen receptor negative tumours (53 cases with37 events)

Variable	nodal status	T stage	Grade	EGFr-2	EGFr-3	EGFr-4	EGFr-c
p-value	0.0327	-	-	-	-	-	-
relative risk	2.2	-	-	-	-	-	-
95% CI	1.1-4.5	-	-	-	-	-	-

In this and the following table (Tables 8.7 and 8.8), the relative risk is the increased risk for each unit increase in that variable (defined in the legend of page 92). The 95% CI is the 95% confidence interval for the relative risk. Relative risk, and its confidence limits, are given only for those variables predictive of outcome.

If, for oestrogen receptor positive cases, overall survival was analysed, EGFr-c remained a significant predictor of outcome: p=0.0006, relative risk, 71.7, 95% CI, 6.2-825.7. Nodal status lost its predictive power with T stage replacing it: p=0.0019; relative risk 2.7, 95% CI, 1.4-5.0.

For oestrogen receptor positive tumours, exclusion of the EGFr-c categorisation did not make any other EGF receptor categorisation predictive of either survival or disease free survival. In a similar analysis of oestrogen receptor negative cases, nodal status remained the only factor that predicted outcome: p=0.0512; relative risk 2.0, 95% CI, 1.0-3.9

Exclusion analyses - nodal status

It is currently accepted practice to establish axillary nodal involvement by sampling or clearance. This has not always been the case and it has been suggested that the features of the primary are paramount in determining prognosis (Harris 1989). This, and the present trend toward more conservative surgery, prompted an analysis of the data excluding data on lymph node involvement. This analysis is presented in table 8.9

Table 8.9: Cox regression analysis of disease specific survival - excluding node status

Breast cancer related deaths (133 cases with 55 events)

Variable	ER status	T stage	Grade	EGFr-2	EGFr-3	EGFr-4	EGFr-c
p-value	<0.00005	0.0158	0.4917	0.5397	0.4780	0.2848	0.2414
relative risk	4.6	1.7	-	-	-	-	-
95% CI	2.7-9.0	1.1-2.6	-	-	-	-	-

Table 8.10: Cox regression analysis of disease free survival - excluding node status

Disease free survival (135 cases with 65 events)

Variable	ER status	T stage	Grade	EGFr-2	EGFr-3	EGFr-4	EGFr-c
p-value	<0.00005	0.0013	0.4226	0.9925	0.9897	0.9527	0.2642
relative risk	3.3	1.9	-	-	-	-	-
95% CI	2.0-5.5	1.3-2.8	-	-	-	-	-

In both tables, the relative risk is the increased risk for each unit increase in that variable (defined in the legend of page 92). The 95% CI is the 95% confidence interval for the relative risk. Relative risk, and its confidence limits, are given only for those variables predictive of outcome.

If all deaths were considered, the same factors were predictive of survival: ER status, p<0.00005, relative risk, 4.1, 95% CI, 2.5-7.0; T stage, p=0.0070, relative risk, 1.7, 95% CI, 1.2-2.5.

Exclusion analyses - oestrogen receptor status

In our series ER status is the most powerful predictor of outcome. This, and the very strong inverse correlation between ER status and EGF receptor levels, made it unlikely that any categorisation of EGF receptor would be significant in an analysis that included ER status. Presented in tables 8.11 and 8.12 are the results of outcome analysis excluding data on ER status.

Table 8.11: Cox regression analysis of disease specific survival - excluding ER status

Breast cancer related deaths (158 cases with 68 events)

Variable	node status	T stage	Grade	EGFr-2	EGFr-3	EGFr-4	EGFr-c
p-value	<0.00005	0.0277	0.0020	0.4926	0.1953	0.1627	0.0505
relative risk	3.3	1.5	1.8	-	-	-	-
95% CI	1.9-5.7	1.0-2.1	1.2-2.7	-	-	-	-

Table 8.12: Cox regression analysis of disease free survival - excluding ER status

Disease free survival (161 cases with 80 events)

Variable	node status	T stage	Grade	EGFr-2	EGFr-3	EGFr-4	EGFr-c
p-value	0.0001	0.0022	0.0060	0.5903	0.4169	0.3691	0.4982
relative risk	2.7	1.7	1.7	-	-	-	-
95% CI	1.7-4.4	1.2-2.3	1.2-2.4	-	-	-	-

In both tables, the relative risk is the increased risk for each unit increase in that variable (defined in the legend of pqge 91). The 95% CI is the 95% confidence interval for the relative risk. Relative risk, and its confidence limits, are given only for those variables predictive of outcome.

If overall survival was considered, these three factors remained predictive and EGFr-c also became predictive: Nodal status, p=0.0002, relative risk, 2.4, 95% CI, 1.5-3.9; T stage, p=0.0043, relative risk, 1.6, 95% CI, 1.2-2.2; Grade, p=0.0039, relative risk, 1.7, 95% CI, 1.2-2.4; EGFr-c, p=0.0314, relative risk, 1.4, 95% CI, 1.0-1.9.

As before if the EGFr-c categorisation was excluded then no other EGF receptor categorisation became predictive.

Analysing for EGF receptor

The aims of this thesis relate to the expression of EGF receptor, therefore some analyses were performed to assess if this variable had a value in predicting outcome. Cox regression analyses were repeated withdrawing the most significant variable at each step. For disease specific survival, oestrogen receptor status and then nodal status had to be excluded before EGF receptor expression became a significant predictor of outcome. Even then T stage and grade were of greater significance. The first categorisation of EGF receptor expression to gain significance was EGFr-c, but if excluded it was replaced by EGFr-4. In an analysis of overall survival EGFr-c was again the first EGF receptor categorisation to gain significance, but only required exclusion of oestrogen receptor status. However, nodal status, T stage and grade were all of greater significance.

For disease free survival the picture was similar. Oestrogen receptor status, nodal status and T stage all had to be excluded before EGF receptor (EGFr-4) expression gained significance. Even then grade had greater significance.

Considering only the categories of EGF receptor expression, the following results were obtained. For disease specific survival, EGFr-3 and EGFr-c were predictive: $p=0.0210$, relative risk, 1.4, 95% CI, 1.1-1.9; $p=0.0583$, relative risk 1.3, 95% CI, 1.0-1.8. For death from all causes, only EGFr-4 predicted outcome: $p=0.0011$, relative risk, 1.4, 95% CI, 1.1-1.6 and for disease free survival it remained the only predictive variable: $p=0.0019$, relative risk 1.4, 95% CI, 1.1-1.6.

Multivariate analysis for local recurrence was not performed as no EGF receptor grouping predicted local recurrence in univariate analyses.

Chapter 9:
Discussion; outcome and clinical parameters

Section i: Relationship to prognostic indicators

Our radioimmunohistochemical results largely corroborate reports in the literature (reviewed in Chapter 2, section ii).

- ♦ There was a strong inverse correlation between EGF receptor and oestrogen receptor expression.
- ♦ Poorer histological grade was associated with higher levels of receptor expression.
- ♦ EGF receptor expression was not associated with T stage.
- ♦ Receptor levels did not correlate with the presence of nodal metastasis.

Much conjecture surrounds the relationship between nodal involvement and receptor expression. In our study, lymph node metastases were not analysed and comment on this aspect of the relationship was not possible. Analysis of all tumours for which nodal status was available hinted at a possible association ($p=0.074$), but this statistic may have been compromised by nodal data for tumours with few or even just one ($n=11$) sampled node. For this reason, a sub-analysis of tumours with 4 or more sampled nodes was undertaken. The choice of four nodes was not accidental; this number provide similar prognostic information to axillary clearance (Steele 1985), and also allowed categorisation into distinct prognostic groups (Miller 1994). However, this sub-analysis simply confirmed that there was no statistically significant association between nodal status and EGF receptor expression.

It appeared, then, that the improved accuracy of radioimmunohistochemistry, compared to conventional techniques, was not advantageous. However, because it provided accurate receptor measurement and did not simply dichotomise tumours to receptor positive or negative, it was possible to perform some further analyses.

EGF receptor is known to mediate a mitogenic signal (Stoscheck 1986, Hamburger 1981, Singletary 1987) and it might seem reasonable that tumour size should correlate with EGF receptor expression. Such a relationship was found when tumour size was analysed in millimetres (Spearman rank analysis, $p=0.049$). A similar finding has been reported (Sainsbury 1987), but using a categorisation of tumour size that was not justified (<15, 16-35, 36-50 and >50mm). Additionally the relationship was reported in terms of EGF receptor positivity rates rather than actual levels of receptor. Using radioimmunohistochemistry a more precise analysis was possible. Although statistically significant, the relationship was not strong (Fig. 8.1). This may be because cellular proliferation is influenced by a number of factors and tumour size will reflect a balance between proliferation and cell loss.

The precision of radioimmunohistochemistry also allowed assessment of the relationship between oestrogen receptor levels and EGF receptor content. By consensus there is an inverse relationship between these receptors, however, this is based on a categorisation of expression of both receptors as positive or negative. Less is known of their relative levels of expression. Koenders *et al* (1991) reported that EGF receptor levels were higher in oestrogen receptor negative tumours than their positive counterparts. Nicholson *et al* (1994), graded immunohistochemical measurement of both receptors as none, low or high, and presented data suggesting that receptor levels might be inversely correlated. Radioimmunohistochemistry enabled this inverse correlation to be corroborated (Spearman rank analysis $p=0.032$). It is worthy of mention that statistical significance, although weak (Fig. 8.2), was apparent only in tumours with oestrogen receptor content measured using immunohistochemistry. Possibly some of the frailties of the EGF receptor ligand binding technique also applied to the ligand protocol used to measure oestrogen receptor.

This inverse relationship is at odds with the suggestion that no phenotype with positivity for both receptors exists (Sharma 1994). Nicholson's group (1994) identified a number of tumours that appeared to have this phenotype but suggested that this resulted from clonal mixing of tumour cells positive for one receptor but negative for the other. Despite this they identified 4 patients in whom endocrine therapy induced complete remissions of tumours 'positive' for both receptors. This they interpreted as indicating either that oestrogen receptor negative, EGF receptor positive cells could respond to endocrine therapy, or that this clone could reverse to an oestrogen receptor positive one. Our results suggested that receptor expression within a tumour was interdependent. If correct, this infers that tumour cells can express both receptors, and also that modulation of expression of one could alter expression of the other. This balance of expression may offer a therapeutic target and is worthy of further study.

Section ii: Adjuvant therapy

Prescription of adjuvant therapy reflected the prevailing clinical practice of the time. Younger patients (pre-menopausal) tended to be prescribed adjuvant chemotherapy whilst older patients (post-menopausal) received tamoxifen. Direct comparison of chemotherapy versus non chemotherapy groups, and tamoxifen with non-tamoxifen groups, was difficult. This was because of the factors that influenced the prescription of these medications. Further, statistical analysis was hindered by the fact that cohorts defined by these treatments were very disproportionate. For these reasons no further analysis was attempted.

Analysis of the prescription of radiotherapy posed similar problems. During the study period, 1984 to 1993, prescribing practice changed considerably. Improved radiotherapy techniques resulted in increasing numbers of patients being referred, particularly after wide local excision, a form of therapy that became more prevalent as the study progressed. The changing approach to the axilla also influenced prescription. Following axillary node sampling those with nodal disease tended to be referred for radiotherapy, but as more axillas were cleared the rate of referral declined, chiefly due to the fear that this treatment combination might induce lymphoedema. This variation in clinical practice, together with some difficulty in determining retrospectively if radiotherapy had been to axilla or chest wall, made more detailed statistical analysis inappropriate.

Section iii: Outcome

Collecting data, and particularly outcome data, in a retrospective fashion is notoriously hazardous. To minimise errors the data were collected by two investigators independently and discrepancies were checked. Compounding this technical difficulty is the clinical problem of determining recurrence, particularly distal but also local. The follow-up surveillance program (mammography, chest x-ray, Pelvic x-ray and liver function tests) was not adhered to religiously, partly as its sensitivity was poor. Additionally, patients were often elderly with significant co-morbidity, and the diagnosis of asymptomatic recurrence was unlikely to significantly modify their management. Not surprisingly, then, in many patients who died breast cancer related deaths, distal metastases had not been diagnosed or were identified only in the terminal phase of illness. Death, because of its unequivocal nature, was, therefore, the most reliable endpoint. In this regard, our study benefited from the confirmed accuracy of the Scottish Cancer Registry (Brewster 1994).

Using the ICD codes deaths were dichotomised to those breast cancer related and those of other causes. Separating deaths in this manner can lead to difficulties since it may be difficult to attribute a particular death to the diagnosis of breast cancer. For example, one patient died in a house fire, the cancer deaths registry did not record breast cancer as a possible cause. However, although an extreme example, it is possible this patient had cerebral metastases and succumbed to these, thereby allowing the fire to escape control. For this reason all deaths, as well as those directly attributed to breast cancer, were considered in the statistical analyses.

a) Characterising the database

Univariate analyses of conventional prognostic indicators were performed to confirm that our patient cohort was representative of breast cancer in general. Both T-stage and histological grade predicted a similar outcome, in our patients, to that observed by others (reviewed by Miller *et al* 1994). Nodal metastases, studied as involvement and as numbers of nodes involved, also produced very similar results. Overall, our patients had a slightly poorer prognosis but this may have related to social deprivation (Schrijvers 1995); the catchment area for Glasgow Royal Infirmary was relatively poor. The only routine prognostic factor which differed to any degree from those in Miller's review was oestrogen receptor. Our patients that were oestrogen receptor positive had a 5 year survival of greater than 80% (as opposed to less than 75%), and our receptor negative patients had an approximately 30% survival (as opposed to 55%).

Multivariate analysis of our data revealed 3 predictors of survival and disease free survival. In descending order of significance, these were oestrogen receptor status, nodal status and T stage. Alderson *et al* (1971) had found the most important factors influencing prognosis, in decreasing order of significance, were, lymph node status, tumour size, stromal reaction and histological grade. Haybittle *et al* (1982) also found lymph node status, histological grade and tumour size predictive. This raises the possibility that our patients are not representative of the general experience of this disease. However, exclusion of oestrogen receptor data made nodal status strongest predictor of outcome and introduced grade as a third predictive parameter. Interestingly, Haybittle (1982) had found the converse, reporting that oestrogen receptor status 'strongly correlated with tumour grade' and, therefore, inclusion of grade had reduced the value of oestrogen receptor data in their multivariate analysis..

The Nottingham prognostic index (Haybittle 1982) and the Yorkshire Breast Cancer Group index make use of a combination of nodal involvement, histological grade and tumour size, weighting their prognostic value in that order. The Nottingham prognostic index has been validated (Todd 1987, Balslev 1994) and can be used to identify good, moderate and poor prognosis groups. In Table 9.1 the Nottingham data (taken from Todd 1987) are compared to our own.

Table 9.1: Comparison of our patients with those from the Nottingham studies.

	% of patient population in each category		
Nodes involved	*A*	*B*	*C*
Nottingham	55	28	17
Our series	49	27	24
Histological grade	*1*	*2*	*3*
Nottingham	18	39	43
Our series	13	38	49
Tumour size (mm)	*<21*	*21-50*	*>50*
Nottingham	49	43	8
Our series	38	49	13
Survival (5 year)	*good*	*moderate*	*poor*
Nottingham	88	69	22
Our series	84	69	21

The Nottingham group assessed nodal involvement using a three node biopsy technique; no nodal involvement (A), involvement of a low axillary node (B) and apical or superior internal mammary node involvement (C). This technique was not employed in our practice and nodes were classified as no nodes involved (A), 1-3 nodes (B) and more than 3 nodes (C) involved (Nodal disease was classified in this way by Balslev *et al* 1994). Modified Bloom and Richardson score (Elston 1991) was used for histological grade. The survival analysis were performed using the Nottingham prognostic index which defines 3 prognostic groups, good, moderate and poor. Kaplan-meier analysis was used to determine five year survival (Kaplan-Meier statistic 49.47, d.f. 2, $p<0.00005$).

In summary, there is a good level of accord. This is true not just of the profile of prognostic factors but also of the survival analysis. The implication is that our patients are reasonably representative of the general experience of breast cancer. For our studies of EGF receptor, this is reassuring.

Why, then, does oestrogen receptor status assume such predictive power in our study? Could our oestrogen receptor data be unusual? In most reports oestrogen receptor positivity rates vary between 50 and 80% (Osborne 1998), in our series it was 56%. Further, of 151 tumours in which this receptor was measured, two different techniques were used for 70 with accord for all but 3 (96%). This implies our data are robust. It has been suggested, as with EGF receptor, that the reported variation of the prognostic value of oestrogen receptor expression may be due to variation in the method of its measurement and the 'cut-off' used to define receptor positivity (Osborne 1998). In the Nottingham series, oestrogen receptor was measured using a dextran coated charcoal method with a cut-off of 5fmol/mg (Haybittle 1982), compared to our cut-off of 20fmol/mg. It has also been suggested that the prognostic value of oestrogen receptor status reduces with duration of follow-up (reviewed in Osborne 1998, Fox 1994). The hypothesis being that it influences the rate of tumour growth but does not correlate to metastatic potential and, therefore, does not determine long term survival. Fox *et al* found oestrogen receptor expression lost its predictive power between 5 and 10 years of follow-up. Median duration of follow-up in our study was approximately 4.5years. Finally, ours is not an entirely unprecedented finding, in a multivariate analysis including lymph node status, tumour size, histological grade, oestrogen receptor status and EGF receptor status, Fox *et al* found only lymph node status and oestrogen receptor status predictive of relapse free survival (1994).

b) Univariate (Kaplain-Meier) analyses

It was our hope that the accuracy of radioimmunohistochemistry would clarify the controversy over the relationship between receptor expression and outcome. In Klijn's review (1992), mean receptor positivity using conventional immunohistochemistry was approximately 42% and with radio-ligand methods was 45%, therefore, splitting the continuum of radioimmunohistochemical results about their median effectively mirrored these techniques. Doing so revealed that EGF receptor expression did predict outcome if death from all causes was the outcome measure, but failed to do so if only breast cancer related deaths were considered. Similar incongruities had been demonstrated in Fox's review (1994), with univariate analysis predictive in 10 of 16 studies. Therefore, despite the greater accuracy of receptor measurement in our study, this analysis of our results only fuelled the controversy.

Conventional measurement techniques designate tumours as receptor positive or negative. However, the radioimmunohistochemical method made it possible to reliably apportion tumours to narrow bands of receptor expression. Dividing the tumours into 3 and 4 groups increased the power of the univariate analysis. Perusal of the survival curves (Figure 8.8) revealed two features:

1. that further divisions more clearly defined a poor risk group, and
2. that survival curves for all but the poorest risk group overlapped.

It was tempting to speculate that the first observation was due to inclusion, in the poorest risk group, of a relatively greater proportion of tumours with amplification of the gene coding EGF receptor. However, amplification rates are very low (Slamon 1987) and only 5 tumours expressed more receptor than normal breast in our study. The statistical impact of so few tumours, in groups of approximately 50 to 100, was unlikely to be large.

The simplest explanation of the overlaps was that EGF receptor levels were poorly predictive of survival, but this ignored the statistical significance of the analysis, and in particular the clear relationship between higher receptor levels and poor outcome. Various explanations were considered, most rational of which may be that small study size had obscured the true relationship.

It was, however, possible that the overlaps were true findings. If this was the case, then moderate levels of receptor expression conferred an initial survival advantage. Could this be a 'time-lag' effect? Also, from the analysis of 4 groups, moderate expressing tumours dichotomised to high expressing good risk and low expressing poor risk tumours. We have demonstrated that, in most breast cancers, expression of EGF receptor and c-*erb*B-2 encoded receptor is interdependent (Robertson 1996). Could these observations reflect receptor interdependency - possibly with EGF receptor a less dominant predictor of outcome than other linked factors? Unfortunately, with the available data, no satisfactory explanation was discernible. Additionally, considering moderately expressing tumours as a separate entity, either temporally or geneotypically, could not be justified on the basis of our data. This group were not distinct in the histogram of EGF receptor expression, nor in terms of their association with other prognostic factors.

For disease free survival, there was also an increase in statistical power as the tumours were divided into more groups. Further, the disease free survival curves (Figure 8.9) followed the same overlapping pattern. This was not surprising. Of the 99 patients with disease recurrence, 81 were included in the analysis of survival and many of the distal recurrences were diagnosed near, or at, the time of death. We might have expected this since disease recurrence, even local (Whelan 1994), is often a harbinger of death. However, our findings were not borne out in the literature. In the reviews of Klijn (1992) and Fox (1994) there was no report that determined an association between EGF receptor expression and survival that did not also find a similar significant relationship to disease free survival. The converse was not true. Fox's, review (1994), larger and more recent, included 16 studies, 10 reported a relationship with disease free survival but only 3 with overall survival. In these studies, follow-up varied not only in duration but also in the manner in which it was reported; mean, median, maximum, and minimum. These studies were, therefore, difficult to compare, but, assuming the disease in each to be similar, an estimate of the study power could be made from duration of follow-up and study size. In the table below an attempt is made to compare those studies that reported a predictive value for EGF receptor, using univariate analysis, that also provided a mean or median follow-up. Our own study (Rihc) is included for comparison.

			follow-up (*t*)	patients (*n*)	*t* x *n*
Predicting DFS only					
	Costa 1988	median	12	376	4512
	Toi 1991	median	31	135	4185
	Osaki 1992	median	32	115	3680
	Spyratos 1990	mean	60	109	6540
	Gasparini 1992	mean	36	164	5904
Predicting Survival & DFS					
	Nicholson 1991	median	45	231	10395
	Koenders 1993	mean	24	376	9024
	Rihc	median	55	194	10670
	Rihc	mean	54	194	10476

Table 9.2 : Studies reporting a predictive value for EGF receptor using univariate analysis. The first column indicates those studies (first author and year of publication) that predicted disease free survival (DFS) or survival and disease free survival. Rihc indicates our radioimmunohistochemical study. Follow-up (*t*) is given in months.

The table shows that a relationship to survival was identified in larger studies with a longer follow-up (greater *t* x *n*). Fox did not report this observation, but it might be expected. Recurrence most frequently occurs in years immediately following primary resection (Karabali-Dalamaga 1978, Willner 1997), however, the time from its diagnosis to death may be protracted (McArdle 1990). This may account for the preponderance of reports of a relationship to disease free survival but not to survival itself. With almost 200 cases and a median follow-up of more than 4.5years, this was not a problem in our study.

On review of the literature, summarised in Chapter 2, no report was found of EGF receptor specifically predicting local recurrence. Although the life tables, in our series, suggested a trend to more frequent local recurrence with higher levels of receptor, none of the statistical analyses was significant. This may reflect the relatively low incidence of this endpoint (48 cases) and a larger study might reveal an association.

In summary, EGF receptor estimation by radioimmunohistochemistry was a significant predictor of survival and disease free survival using univariate analysis. The next goal was to determine if it could predict outcome in a multivariate analysis, and thereby provide an advantage in the clinic.

c) Multivariate (Cox regression) analyses

In multivariate analysis that included oestrogen receptor status, lymph node status, histological grade, tumour size (TNM) and EGF receptor expression, no categorisation of EGF receptor levels was significant.

Conventional prognostic indicators, particularly nodal status, are used to determine adjuvant therapy. Identification of high or low risk groups within these conventional subgroupings could be useful when planing treatment. For this reason our data were analysed in subgroups. Published reports of such analyses (reviewed in Chapter 2 section iii) have been contradictory. This is particularly the case for sub-analyses based on oestrogen receptor status, but less so for lymph node negative disease, where EGF receptor may have a predictive value (Sainsbury 1987, Nicholson 1990, Spyratos 1990, Fox 1994). In our series, EGF receptor expression failed to determine outcome in either node negative or positive subgroups. A similar analysis of oestrogen receptor positive, and negative, disease showed that only in the former did EGFr-c have predictive power. Clinically this categorisation was not very useful since 97% of tumours, those expressing less than normal breast levels of EGF receptor, were classified together.

Therefore, if routine prognostic indicators were available, EGF receptor measurement provided no clinical advantage. However, Harris *et al* (1989) had pointed out that nodal information was not always available. In our study, exclusion of lymph node status did not make EGF receptor predictive. Although more difficult to justify, a similar analysis was performed excluding oestrogen receptor status. Radioimmunohistochemistry had revealed a strong inverse relationship between oestrogen receptor status and EGF receptor expression, it was, therefore, unlikely that both factors would be significant in a multivariate analysis. Without oestrogen receptor data, EGF receptor predicted deaths from all causes but not breast cancer related death or disease free survival. Only the EGFr-c categorisation was predictive but was less so than nodal status, T stage and grade. As indicated above this categorisation of EGF receptor has little clinical value. If not included in the analysis then no other EGF receptor categorisation replaced it. Overall, EGF receptor expression was the least significant predictor of outcome in our multivariate analysis.

In summary, radioimmunohistochemical measurement of EGF receptor did not provide useful prognostic information using a multivariate analysis that included routine prognostic factors.

PART 3

EGF RECEPTOR
AND
BREAST CANCER CELL INVASION

Overview of invasion studies

This second experimental part of the thesis takes cognisance of the documented influence of EGF receptor on breast cancer prognosis and explores the possibility that this relates to an effect on tumour cell invasion.

The assay used for these invasion studies had not previously been reported and the first subsection (Chapter 10) documents the problems overcome in its development and provides evidence of its reproducibility. The second subsection (Chapter 11) includes the results of the invasion studies for the different breast cell lines. It also includes experiments that confirm that invasion is EGF receptor mediated. Finally, efforts were made to abrogate the invasive response using agents with therapeutic potential.

Chapter 10:
Developing the invasion assay

Section i: Available tissues

Since the intention of this work was to try and ascertain the role of EGF receptor in human breast cancer, cell lines established from human breast cancers were studied. These were; MCF7, ZR75.1, SKBR3, MDA-MB-361, MDA-MB-453, MDA-MB-231, BT20, MCF7ADR (see Table 10.1). All the lines were available locally (Institute for Cancer Research at the Cancer Research Campaigns Beatson laboratories, Garscube Estate, Glasgow) and, with the exception of MCF7ADR, were available from the American Type Culture Collection (ATCC). The MCF7ADR cell line was produced by culture of MCF7 cells with increasing amounts of Adriamycin (Vickers 1988). The passage number of the cell lines was not available but was likely to be high.

Cells were maintained in culture medium, Dulbeccos Modified Eagle's Medium (DMEM) or RPMI 1640 (RPMI), composition shown below. The MDA-MB-361, MDA-MB-453 and BT 474 lines were maintained in DMEM. The remaining lines were grown in RPMI. Both media were supplemented with 10% foetal bovine serum and the cells were cultured in 95% air, 5% CO_2 at 37°C. Flasks were grown to sub-confluence and cells were then split using a trypsin solution. In this manner the cell lines were maintained until completion of the experimental work.

Section ii: Materials

Growth factor reduced Matrigel® (Collaborative Research, Becton Dickinson) is a soluble basement membrane extract of the Engelbreth-Holm-Swarm tumour, it is liquid at -20^{o}C but solidifies at room temperature (Kleinman 1986). The main components of Matrigel are laminin, collagen IV, entactin and heparan sulfate proteoglycan (Kleinman 1982, Bissel 1987). Growth factor reduced Matrigel®, as its name implies, has reduced concentrations of growth factors compared to Matrigel® (see Table 10.3). Since these include EGF, it was used for the experiments presented below and is referred to hereafter as Matrigel. Matrigel matrix has been used extensively as an *in-vitro* barrier to study the metastatic potential of tumour cells (Taniguchi 1989, Terranova 1986, Albini 1987, Hendrix 1987).

Table 10.1: Characteristics of the studied cell lines.

Cell line	Origin	Morphology *in-vitro*
BT20	Primary	Typical adenocarcinoma
BT474	Primary	Epithelial, multi-layered colonies
MCF7	Pleural effusion	Epithelial like
MCF7ADR	Pleural effusion	Epithelial like
ZR75.1	Ascitic effusion	Adenocarcinoma
MDA-MB-231	Pleural effusion	Anaplastic adenocarcinoma
MDA-MB-361	Brain metastasis	Epithelial like
MDA-MB-453	Pleural / pericardial effusion	Epithelial like patches, single spheres
SKBR3	Pleural effusion	Adenocarcinoma
3T3*	Mouse embryo	Fibroblast-like

* This cell line was used to produce fibroblast conditioned medium for use as a chemoattractant.

Table 10.2: Composition of the tissue culture media

	DMEM	RPMI
Sterile water	800ml	800ml
DMEM (x10 conc.)	100ml	-
RPMI (x10 conc.)	-	100ml
Foetal calf serum	100ml	100ml
Glutamine (200mM)	10ml	10ml
Pyruvate (100mM)	10ml	10ml
Sodium Bicarbonate (8.4%)	40ml	40ml
Hepes Buffer (1M)	-	20ml
Sodium Hydroxide (1M)	8ml	-
Steptomycin / Penicillin	10ml	10ml

Table 10.3: Composition of reduced growth factor Matrigel

Constituent	Growth factor reduced Matrigel
EGF (ng/ml)	<0.5
bFGF (pg/ml)	0-0.1
IGF-1 (ng/ml)	5
PDGF (pg/ml)	<0.2
TGF-β (ng/ml)	107
% protein that gels	83

Costar's Transwell™ cell culture chamber inserts were used as assay chambers (Catalogue No. 3422, Costar Europe Ltd.) (see Figure 10.1). Sterile 24 well cluster plates were supplied with 12 inserts. These inserts have a polycarbonate membrane base which is suspended above the well floor by a flange at the top of the insert. The polycarbonate base is 6.5mm in diameter and has pores of 8µm diameter, this is referred to hereafter as the filter. The remainder of the construction is transparent polystyrene. The lower part of the wall of the insert is non-porous; thus the filter separates a 300µl upper compartment (within the insert) from a lower 1000µl compartment (the well). Air can circulate from the insert to the well via holes in the upper part of the insert.

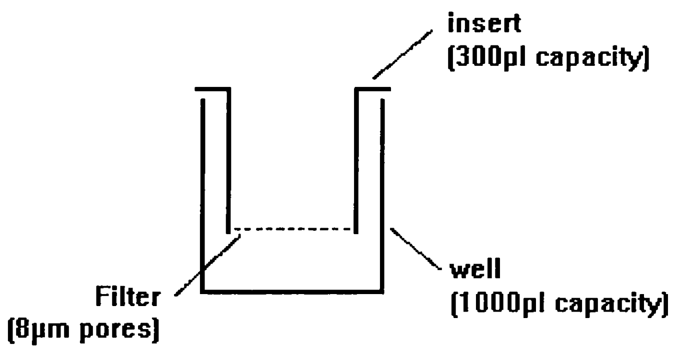


Figure 10.1: Schematic cross section of a Costar Transwell cell culture chamber

Section iii: Establishing the Invasion assay

The invasion assay used in this series of experiments was a modification of an assay which had been developed under guidance from Dr. Bradford Ozanne (Hennigan 1994). This assay differed from those previously reported, in that;

1. it utilised a thick layer of Matrigel,
2. assay analysis was performed on an intact 3-dimensional Matrigel layer.

A full description of the assay protocol, as used for these breast cell line studies, is provided at the end of this chapter, but to facilitate understanding of the experiments that led to this protocol, a brief description of the original, as described by Hennigan (1994), is given here. Matrigel was added to the Costar Transwell inserts and allowed to gel. The inserts were then inverted and a suspension of the cells under study was added to the filter base. The gelled Matrigel did not slip out of the insert and the cells applied to the filter bottom adhered to it. The inserts were then inverted again, returning them to their original orientation, and were placed in the Costar Transwell wells, to which had been added culture medium. Chemoattractant in culture medium was then added above the Matrigel layer. After incubation, the assay was fixed using ice cold acetone, cells were stained with propidium iodide, and the assay was read using the confocal microscope.

The original assay protocol was developed to assess the invasive capacity of *fos* transformed fibroblasts and required considerable modification before it could be applied to the breast cancer cell lines under study in this work. This chapter reviews some of the experimental work which led to the final invasion assay protocol. What follows is a description of the experiments that justified these modifications and the problems that these overcame. Some assays were repeated when protocol modifications were introduced, others were not. Many of the assays addressed more than one issue and some were used to corroborate other results. Some results in this section may seem contradictory but this reflects the changing protocol. The series of assays presented in Chapter 11 were all performed using the final assay protocol. The experimental results used to produce the plots in chapters 10 and 11 are contained in Appendix 3, results for any particular experiment are in tables named after the plots they were used to produce.

a) Problem - would EGF stimulate invasion in this assay?

The aim of the initial experiments was to determine if the assay system could be used to study an invasive response stimulated by EGF.

Experiments

A variety of chemoattractants were added to the upper chamber of the assay system. The results of these first experiments are summarised in the plot below.

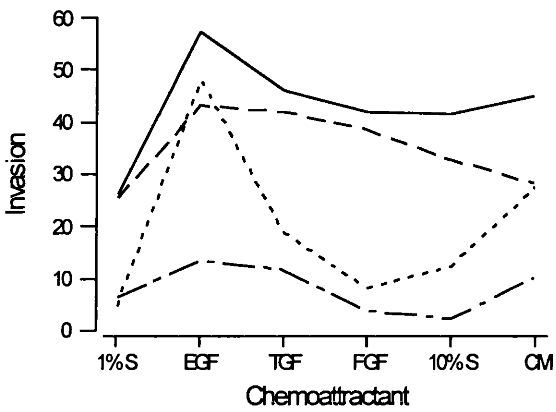


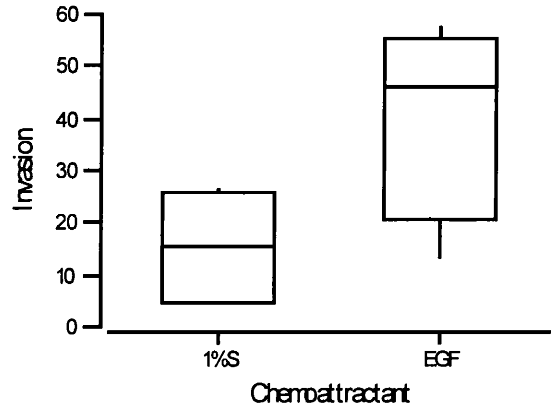
Figure 10.2: Invasion using different chemoattractants

This plot shows the results for the MDA-MB-231 cell line. Each of the lines represents a separate assay. The y-axis shows invasion expressed as the sum of the percentage fluorescing pixels in each of six optical sections. Readings were taken in 5μm steps, from the optical section judged to be at the level of the filter, for 25μm (6 optical sections). The x-axis shows the chemoattractant placed above the Matrigel layer. Culture medium with 1% foetal calf serum was used as a control (1% S). The chemoattractants were; culture medium augmented with 20ng Epidermal Growth Factor (EGF), 1ng Transforming Growth Factor-βeta (TGF), 10ng acidic Fibroblast Growth Factor (FGF), 10% foetal calf serum (10% S) and fibroblast conditioned medium (CM).

Whilst the plots for each assay followed similar profiles the magnitude of invasion varied. This is a problem which is addressed later in this section.

These data suggested that addition of EGF above the filter stimulated cells to invade into the Matrigel layer (Figure 10.2). A similar effect was suggested for TGF and fibroblast conditioned medium. The results for both 10% foetal calf serum and acidic fibroblast growth factor were more difficult to interpret.

Figure 10.3: Using 1% foetal calf serum and 20ng EGF as chemoattractant.



The x-axis shows the chemoattractant placed above the Matrigel layer; 1% foetal calf serum (1%S) and 1% foetal calf serum augmented with 20ng EGF (EGF). On the y-axis is invasion. This is shown as the mean and interquartile range of the sum of the fluorescing pixels, from the 4 experiments plotted above. Analysis of variance gives a p value of 0.067.

The results of Analysis of Variance for each of the tested chemoattractants versus 1%S (designated the control) are shown below.

Chemoattractant	n	mean	St. Dev.	p
1% S	4	15.62	11.77	-
EGF	4	40.55	18.95	0.067
TGF	4	27.67	14.29	0.241
FGF	4	23.05	19.98	0.546
10% S	4	29.62	17.00	0.224
CM	4	22.23	18.21	0.565

Conclusions

The initial experiments suggested that EGF might stimulate breast tumour cell invasion. It is true that a similar effect with the other chemoattractants could not be excluded on the basis of the data presented; only one concentration of each chemoattractant was used (albeit a concentration chosen after careful consideration and perusal of the literature). However, the chief interest of the investigator was the role of the EGF receptor and the cost, both in time and materials, of pursuing experiments with other chemoattractants, in an assay system that was still not proven, was considered unjustifiable.

b) Problem - what control to use?

Without some foetal calf serum there was concern that cells might not survive over the incubation period (7 days), but foetal calf serum contains a variety of factors which could influence the assay. Parish *et al* (1992) reported that exclusion of foetal calf serum from the assay did not significantly alter the invasion of rat mammary carcinoma cell line 13762 MAT. They interpreted this to mean that foetal calf serum did not contain enzymes that had a role in basement membrane degradation. However, Imamura *et al* (1991) found that without serum supplement there was marked reduction of invasiveness of both rat hepatoma cell line, AH130, and human small cell lung cancer line, OC10.

Experiments

A range of different serum concentrations were studied to ascertain the optimal concentration for the final assay protocol (see Figure 10.4).

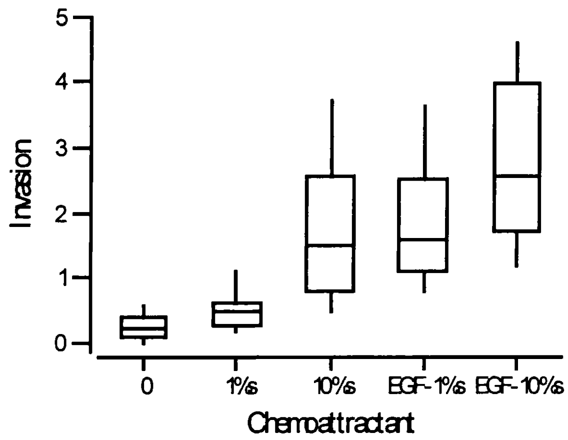


Figure 10.4: Determining a control for the invasion assays

Results shown are for the MDA-MB-231 cell line. Data is depicted as the mean and interquartile range. Invasion, plotted on the y-axis, is shown as the mean and interquartile range of five readings from each of two assays. Only the percentage fluorescing pixels in the optical section at the level of the filter was determined. Shown on the x-axis is the medium used for the assay; culture medium with no foetal calf serum (0), culture medium with 1% foetal calf serum (1%s), culture medium with 10% foetal calf serum (10%s), culture medium with 1% foetal calf serum and 20ng EGF added above the Matrigel (EGF-1%s), culture medium with 10% foetal calf serum and 20ng EGF added above the Matrigel (EGF-10%s).

Though cells in the Matrigel layer are difficult to assess using light microscopy, those that fall from the filter base into the lower assay chamber can be studied. These cells were poorly viable at the end of incubation if no serum supplement was used. Using medium augmented with 1% foetal calf serum, cells remained viable and invasion was minimal in the absence of EGF.

Conclusion

Culture medium with 1% foetal calf serum was used as a control.

It is worth reiterating that the experiments used to establish the assay protocol were not always analysed in the same manner - reflecting the development of the assay protocol. Additionally, the experiments are not presented in strict chronological order. This has resulted in variation in the manner of data presentation. On this occasion, invasion is recorded as fluorescence at the level of the filter alone (preceding figures showed invasion as the sum of fluorescence in a stack of optical sections). To minimise confusion, each plot is accompanied with a comprehensive description of what is presented.

c) Problem - which cell lines could be studied?

The next experiments were to determine which cell lines would be suitable for study using this assay. Results are summarised in Table 10.4. The recorded results are of the best invasive response to EGF. Morphology refers to appearance of cells in the assay wells, viewed with conventional light microscopy prior to fixation, and cells in the inserts, viewed post fixation, with fluorescent light microscopy/confocal microscopy.

Conclusion

It was evident that some cell lines would not cross the filter using this assay system and others would only cross the filter but did not seem to invade into the Matrigel layer. However, there were no cell lines that crossed the filter in response to a chemoattractant other than EGF that did not also respond to EGF. This observation is open to the criticism voiced above; that a failure to respond to a single concentration of chemoattractant does not exclude a response at different concentrations. However, as before, the aim of this work was to assess the EGF receptor and these early experiments did not suggest that these other chemoattractants were likely to induce invasion that could then be modulated by EGF binding of EGF receptor.

Table 10.4: Characteristics of the cell lines studied in the invasion assay.

cell line	morphology	Invasion*
MDA-MB-231	Cells, with pseudopods extending, were seen high in the Matrigel layer. Below the filter there was a confluent layer of cells.	+++
MCF7ADR	Cells grew in mounds but some invasion into the Matrigel was evident. Below the filter there was a confluent layer of cells.	++
BT20	Some cells crossed the filter and some were apparent above the filter. Cells below the filter were subconfluent.	+
MDA-MB-361	Some cells crossed the filter but there was no convincing evidence of invasion into the Matrigel. Cells below the filter were subconfluent.	+
SKBR3	Few cells crossed the filter. Cells below the filter were poorly subconfluent.	+
MDA-MB-453	No cells were above the filter. Mounds of cells grew down below the filter.	-
ZR75.1	No cells were above the filter. Cells were confluent on the underside of the filter	-
BT474	No cells were above the filter. Mounds of cells grew down below the filter.	-
MCF7	No cells were above the filter. Cells were confluent on the underside of the filter.	-

Table 10.4 (continued): Characteristics of the cell lines studied in the invasion assay.

* Invasion increased from '+' to '+++', no invasion is indicated by '-'

d) Problem - could invasion be increased using an altered assay orientation?

Unlike fibroblasts in the original studies, breast cancer cells invaded to a limited extent. If a greater invasive response could be induced then this would be easier to study. The protocol required plating of tumour cells onto the bottom of the filter and Matrigel above it. The filter, so interposed, could itself be an obstruction to invasion. Experiments were, therefore, undertaken to try and modify the invasion assay in order to place a monolayer of study cells in direct contact with the Matrigel layer.

Experiments

Several approaches were adopted. Cells were plated onto the upper surface of the filter and Matrigel was added above this layer. This proved unsatisfactory as even careful application of the Matrigel resulted in cells becoming dislodged from the filter surface and trapped within it. Even allowing the cells to adhere to the filter overnight did not obviate this problem.

An alternative approach was to add cells above the Matrigel layer (with the chemoattractant in the assay well). This was unsuccessful as the cells failed to form a monolayer over the Matrigel but clumped together at the lowest point of the Matrigel meniscus. This may, in part, be due to gravity but there is also evidence to suggest that breast cells grown on Matrigel form lobuloalveolar-like aggregates (Streuli 1991, Darcy 1991).

In a further experiment, Matrigel was applied, and allowed to gel, to the underside of the filter with the study cells then added to the bottom of this layer. This also proved unsatisfactory as the cells seemed reluctant to adhere to the Matrigel and, when excess cell suspension was removed from the Matrigel coated filter bottom, the Matrigel was in danger of becoming dislodged.

Finally, since it was possible that cells might occlude the filter pores (Parish 1992), preventing, or reducing, invasion, filters with larger, 12μm, pores were tried. However, cell lines that failed to cross the 8μm pores would not cross the 12 μm pores, a finding in concordance with the reports of others (Simon 1992).

Conclusion

The premises of these experiments had been:

1. to induce an invasive response in the cell lines that would not cross the filter using the original assay protocol,
2. to determine if removing the filter barrier would increase the invasive response in those cell lines that did cross the filter.

Neither objective was achieved and despite the paucity of cell lines that could be studied, the original assay orientation using filters with 8μm pores was adopted for the remainder of the invasion studies.

e) Problem -what was the significance of the EGF concentration gradient?

Since EGF is a relatively small molecule, 53 amino acids, and the assay was incubated for 7 days, it was probable that a concentration gradient existed for only a part of the total incubation time.

Experiments

To ascertain the significance of the concentration gradient a series of experiments was conducted comparing the effects of placing EGF in the upper and lower assay chambers. The results are shown in the Figure 10.5.

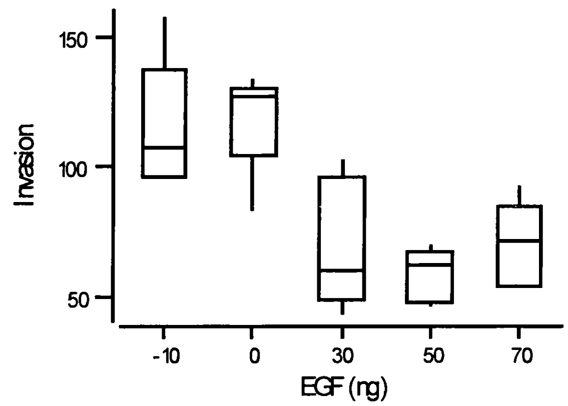


Figure 10.5: Determining the significance of the concentration gradient.

This boxplot shows the mean and interquartile range. Invasion, on the y-axis, is presented as the sum of the pixels above the filter (8 optical sections). The x-axis depicts the amount of EGF added; '-10' indicates that 10ng EGF was added above the filter, in the remaining assays EGF (ng) was added to the lower assay chamber (well). The lower assay chamber volume was approximately 3 times that of the upper chamber.

Conclusion

This plot suggested that the chemoattractant placement was important, placing it above the Matrigel appeared to generate a concentration gradient which did influence the extent of tumour cell invasion.

f) Problem - what was the significance of the Matrigel meniscus?

The viscous Matrigel produced a visible meniscus; thicker at the periphery than at its centre. As the preceding work had demonstrated the EGF concentration gradient influenced the degree of Matrigel invasion. The natural syllogism was that invasion would vary across the meniscus.

One method, for reducing the meniscus effect, was to coat the inside walls of the insert with a water repellent. This technique had been described by Imamura *et al* (1994). They rubbed a block of paraffin wax on the inner wall of the inserts of Chemotaxicell chambers, 8mm diameter (8μm pores), (Kubota Co., Toyko, Japan), using cotton to remove any excess. Using this method, they demonstrated that a reduced meniscus depth had an effect on the invasive response. This they achieved by adding erythrocytes (diameter 7μm, filter pores 8μm) to the upper assay chamber and counting those that crossed the filter. Wax, so applied, reduced by 5-fold the number of erythrocytes crossing a filter coated with a 70μl aliquot of 0.2mg/ml Matrigel protein. The explanation of this observation was that the central portion of the Matrigel barrier was deficient. In our protocol, this problem was potentially compounded since the filter insert was inverted to seed test cells onto its underside; an attenuated or deficient Matrigel layer might allow cells to cross the filter at this stage.

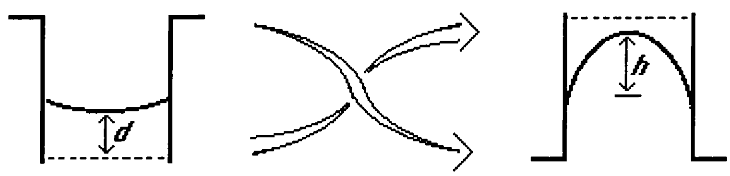


Figure 10.6: Schematic of the Matrigel meniscus and the effect of gravity. Inversion increased the height (h) of the meniscus and reduced the depth (d) of the Matrigel layer.

Imamura also reported that the magnitude of the invasive response varied across the meniscus; describing a uniform pattern of cells crossing the filter when the meniscus was minimised, as opposed to a central preponderance without wax application. In our protocol, the pattern of invading cells seemed relatively uniform, however, formal assessment of this potentially serious problem was required.

Experiments

By reducing the Matrigel concentration it was possible to reduce the meniscus curve, therefore, assays with reduced concentrations of Matrigel protein were tested. However, dilutions of greater than 1 part Matrigel to 2 parts culture medium failed to remain adherent when the insert was inverted for cell seeding. For the subsequent studies a ratio of 1 volume Matrigel to 1 volume medium was used.

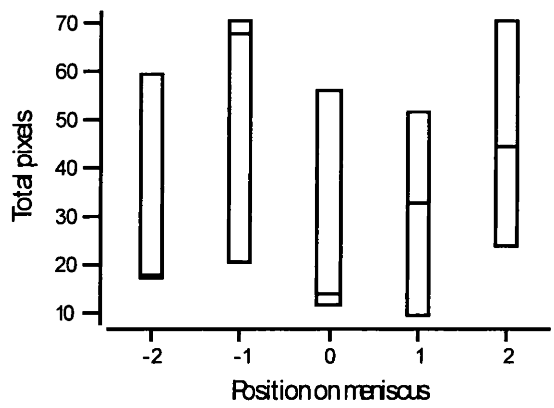
The next experiment addressed the possibility that cells crossed the filter whilst it was inverted. Several inserts were set up but, after seeding cells and before reverting the insert, the filter was wiped to remove cells on its underside. Any cells which had crossed the filter would not be effected by this manoeuvre. Control filters were not wiped. Wiped, and unwiped, filters were then incubated with 10ng EGF as a chemoattractant. No cells were evident, in the wiped assays, at one week. Thus cells did not cross the filter during the period of cell seeding.

Imamura had reported that, with 100μl of 1mg/ml Matrigel protein, no erythrocytes crossed the filter. Using our protocol, 80μl of approximately 5mg/ml Matrigel protein was used; approximately 4-times more than that which produced zero erythrocyte counts in Imamura's study. This, in conjunction with the experiment above, suggested that the Matrigel layer was not deficient.

Next, a Pap pen, instead of wax, was used to reduce the meniscus depth. Although macroscopically effective there were misgivings. In our hands it proved technically difficult to apply the repellent to the well sides evenly without also applying it to the filter surface. This might jeopardise the uniformity of the assays, a prime advantage of the Matrigel assay model. Further, there was concern that the process of repellent application might lead to an increase in infected well systems or that the repellent itself might effect, either, the Matrigel or the study cells. Because of these concerns, and hypothesising that the central preponderance of invasion reported by Imamura (1994) reflected a deficiency in the Matrigel layer which did not occur with our protocol, experiments were performed to assess the significance of the meniscus in our protocol.

A number of filters were studied across their diameter. A millimetre scale was placed, on the confocal microscope stage, adjacent to the filter. The centre point of the filter was determined grossly and readings were taken from this point (0) and, using the microscope stage controls to move the stage in one plane only, readings were taken from 1 and 2mm either side of this central point. This experiment was performed in triplicate and results are shown below.

Figure 10.7: Determining the significance of the Matrigel meniscus



On the x-axis, invasion is depicted as the sum of pixels in 6 optical sections. The y-axis shows the position across the meniscus from which the readings were taken. Data is presented as the median and 95% confidence limits. Analysis of Variance p=0.66.

The results revealed no significant variation in invasive response across the meniscus. This clearly contradicts Imamura's results, but probably reflected both the low protein content of his barrier and his own evidence that this layer could be deficient. That there is no effect on the invasive response in our protocol, maybe because EGF placed above the Matrigel equilibrates through it, reducing the significance of concentration gradient in any part of it. (The preceding experiment revealed that placing the EGF above the Matrigel encouraged most invasion, this may have been because the EGF adhered to the Matrigel components.) Another possibility was that the depth of the Matrigel meniscus, obvious with a Matrigel/air interface, was reduced by the addition of chemoattractant containing culture medium above the Matrigel (the depth of the meniscus at the interface of two fluids relates to their relative densities, personal communication from B Scott, Professor of Mechanical Engineering, University of Glasgow).

Immediately adjacent the periphery of the filter the optical image did appear distorted. The cause of this problem was not formally elucidated but was attributed to a 'meniscus effect', although an optical component, from the polystyrene wall of the filter assembly, may have contributed. It was decided to avoid accumulating data from points at the immediate periphery of the filter.

Conclusions

The Matrigel layer provided an intact barrier to invading cells. To ensure uniformity in the assay system, and to avoid the problems outlined above, the presence of a meniscus was accepted. The final assay protocol required that readings were taken from around the central portion of the filter. Fields were selected in a random manner; between readings, the filter assembly was moved slightly without visualising the selected field. Three to five fields were analysed for each assay. The reliability of the assay was based on replicating measurements (Mackinnon 1992).

g) Problem -what was the optimal incubation period?

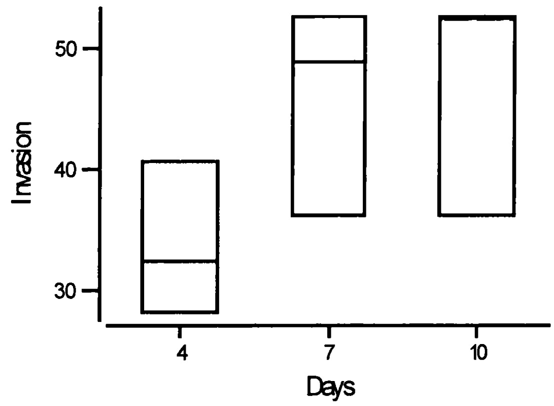
Albini's (1987) original Matrigel/Boyden chamber assay was described as 'rapid *in-vitro* assay' and the short incubation period, 6 hours, was considered to be one of its merits. Subsequent modifications of the assay system have included more prolonged incubation periods, up to 72 hours (Repesh 1989, Imamura 1994). In all reported protocols, the quantity of Matrigel applied to the filter has been considerably less than in our protocol, therefore, a further series of experiments was conducted to determine optimum incubation period.

Experiments

Since duration of incubation could alter the degree of invasive response, cell lines that did not cross the filter, as well as those that did, were studied. Initially assays were incubated for 4, 7 and 10 days. Results for the MDA-MB-231 cell line are shown below.

Cells in the Matrigel layer are difficult to visualise using light microscopy, however, cells accumulating in the wells, having fallen from the filter, could be assessed in this manner. In assays incubated for 10 days, examination of these cells revealed that many were dead. Repeating the assays, this time replacing the medium in the well at 7 days, reduced this problem but prolonging the incubation from 7 to 10 days did not greatly increase invasion and there was an increased attrition, with assay chambers succumbing to infection. Additionally, cell lines that failed to invade with a 7 day incubation did not invade with a prolonged incubation.

Figure 10.8: Altering the duration of the assay incubation.



Invasion, on the y-axis, is represented by the mean and interquartile range of the sum of fluorescing pixels in 6 optical sections. The x-axis indicates the number of days of incubation before the assay was analysed.

Conclusion

For the remainder of the studies an incubation period of 7 days was used.

h) Problem - how best to collect optical sections?

Using the original assay protocol and fibroblast cell lines, invasion of up to 80μm was detected (Hennigan 1994). The maximum invasion of the breast cell lines was much less, approximately 30-40μm. For this reason the step between optical sections, which had been 5μm in the original assay, was reduced to 3μm. This in turn generated another problem; with invasion extending for only 20-30μm small errors in determining the filter (0) level could have profound effects on interpretation of the extent of invasion.

Difficulty in determining the base level stems from the fact that the filter itself was not, or was only poorly, visible using fluorescent microscopy. Marking the filter with a fluorochrome was one possibility, but there would then be a risk that this chemical might influence the assay (it would need to be applied prior to setting up the assay), or that its application might damage the fragile filter. This problem was solved by what came to be known as the 'bottom wipe'. This involved gently wiping the bottom of the filter with lens cleaning tissue. This action removed all the cells below the filter, thereby providing two advantages;

1. that the level of the filter could be determined, accurately and reproducibly,

2. that fluorescence from cells below the filter would not compromise measurement of fluorescence from invading cells.

Experiments

Three assays (1-3) were analysed both unwiped, 'u', and wiped, 'w'. The same field was analysed 5 times, each time the filter (0) level was reassessed.

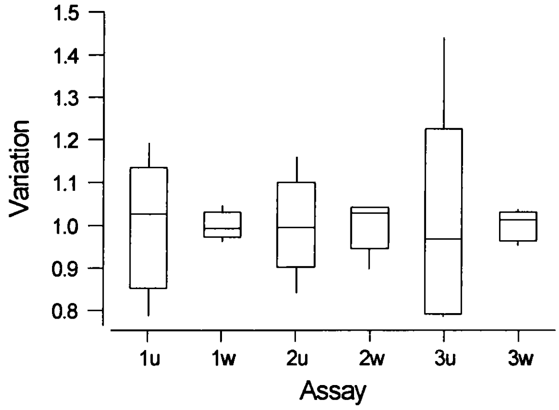


Figure 10.9: Wiping the filter bottom improves assay accuracy

The x-axis shows the assay number (1-3) and also whether the readings were taken from the well before wiping (u) or after wiping (w). Variation, y-axis, is reported as the % of field pixels fluorescing at the filter (0) level divided by the mean of this value for the five readings. Results for each assay are shown as median and interquartile range.

Conclusion

Figure 10.9 clearly shows that wiping the bottom of the filter reduced variation in the assay analysis. This technique was, therefore, adopted for the final assay protocol.

i) Problem - how could different assay results be compared?

Assessment of the results of the early experiments revealed that pixel counts, reflecting cell numbers, in each optical section varied for experiments that were intended to be identical. Overall trends for the experimental series would be similar but pixel values could be quite different (evident in Figure 10.2: Invasion using different chemoattractants). There were a number of possible causes for this observation - EGF that was thawed and re-frozen might be less efficacious in later assays; protein content of the Matrigel might vary; pressure on confocal microscopy time meant that the time between assay fixation and assay reading varied, etc.

Conclusion

Because of these difficulties, and following the precedent of Parish *et al* (1992), results for repeated assays were standardised. This meant that results from each assay series would assume equal significance, facilitating data accrual to strengthen particular arguments and allowing results from different assay series to be directly compared. The final protocol required that results be standardised so that maximal invasive response was recorded as 1 and the minimum as zero.

Section iv: Interpretation of assay results

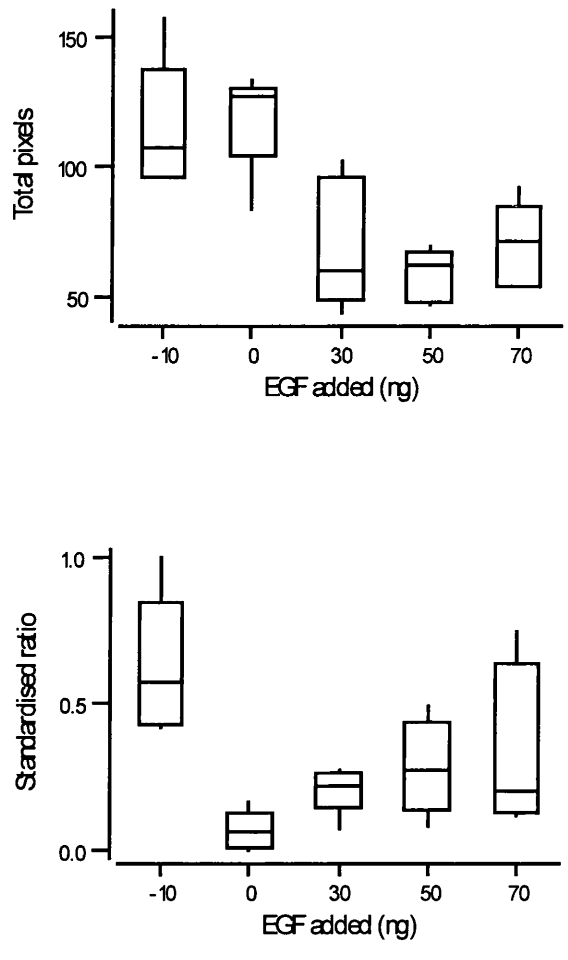
There were no previous reports of the use of confocal microscopy, to measure tumour cell invasion in a thick Matrigel layer. Using this technique, very thin optical sections were analysed and the image analysis software recorded the percentage of the confocal field that fluoresced above a selected intensity. Propidium iodide was used as the fluorochrome because it adhered to DNA but did not attach to the Matrigel, filter or polystyrene insert. The fluorescence threshold was selected so that cells were clearly visible but background noise was minimal. Thus the percentage of the confocal field that fluoresced above this threshold was proportional to the number of cells in that section. Maintaining the fluorescence threshold for all the inserts in a given assay allowed results for each insert to be directly compared.

Cells that crossed the filter were considered to have demonstrated an invasive response. The sum of the pixels, in each optical section, above the filter was considered to reflect a combination of invasion and proliferation. Unlike previously reported assays, our protocol could be used to assess the progression of cells within the Matrigel layer. This could usefully be measured by comparing the number of cells at higher levels in the Matrigel layer with those at lower levels. This ratio was considered to be indicative of the degree of tumour cell invasion induced by the chemoattractant.

Whilst this ratio proved useful in interpreting some invasion assays, care in its use was required. If there was very little invasion in an assay the ratio could become misleading. Consider an extreme example; if there is no invasion, then the sum of pixels at lower and higher levels in the Matrigel layer will have the same background count, the ratio is then one (background over background). For this reason it was important to calculate the total sum of pixels above the filter and on some occasions determining the ratio was not helpful. Occasionally, interpreting results of one method was facilitated by the results of the other.

Consider Figure 10.10, produced from the experimental results shown in Figure 10.5: Determining the significance of the concentration gradient.

Figure 10.10: Comparing the methods of assay interpretation.



In both plots, the x-axis indicates the amount of EGF (ng) added to the assay; EGF was added to the well, that is below the filter, except for the assays indicated by the negative precursor, where the EGF was added above the Matrigel. The y-axis depicts invasion but this is measured in different ways in each plot. In the upper plot, it is the sum of fluorescing pixels above the filter, in the lower plot invasion is represented as a ratio of fluorescing pixels higher in the Matrigel layer over pixels at lower levels in the Matrigel. In both boxplots data is shown as median and interquartile range.

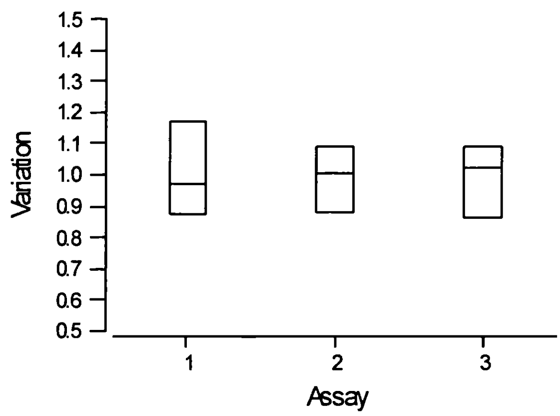
In the upper plot, the y-axis shows the sum of pixels in all optical sections above the filter. The lower plot presents the results for the same assay but shows the ratio of pixels in higher optical sections over those in lower sections, termed hereafter, the standardised ratio.

These plots suggested that similar numbers of cells crossed the filter in the control (0) assay as did with EGF added above the filter (-10), but those cells in the latter had invaded further into the Matrigel layer. The caveat indicated above probably applied to the wells with EGF added below the filter; there were few cells crossing the filter causing the ratio to be misleading.

Section v: Reproducibility

It is obviously important to establish the reproducibility of both the experimental results and the assay technique. The latter was addressed by repeatedly reading a single field in one insert. Between taking each series of readings the microscope stage was moved down and the base (0) level re-established as described above. The results confirmed that the method of reading an assay insert was reproducible (Figure10.11).

Figure 10.11: Reading the same confocal field.

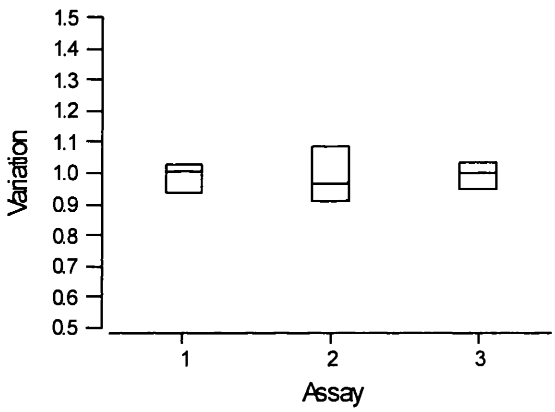


The x-axis indicates the assay analysed. The y-axis shows the variation in the sum of pixels measured in each of 5 assays of a single confocal field. To make the graphic representation of this data simpler results for each assay are standardised by dividing by the mean. The plot shows the median result and the 95% confidence limits. The table, below, shows the un-standardised results.

Assay	Readings	mean	Std. Dev.
1	5	55.5	6.26
2	5	43.7	3.31
3	5	39.2	3.33

The reproducibility of the optical analysis was assessed by a similar experiment on three different assays (figure 10.12). Having identified the filter (0) level, the series of optical sections was collected on 5 separate occasions, the results show 95% confidence limits for the standardised sum of pixels above the filter (8 confocal sections, 0-21μm). There was clearly very little variation.

Figure 10.12: Reading the same optical section series.

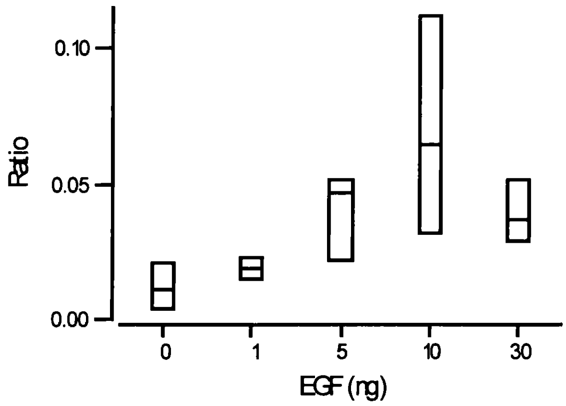


The x-axis indicates the assay analysed. The y-axis shows the variation in the sum of pixels measured in each of 5 analyses of the same stack of optical sections. To make the graphic representation of this data simpler results for each assay are standardised by dividing by the mean. The plot shows the median result and the 95% confidence limits. The table below shows the un-standardised results.

Assay	Readings	mean	Std. Dev.
1	5	88.1	3.26
2	5	58.7	4.86
3	5	89.9	2.99

The reproducibility of the experiments was confirmed by performing repeat experiments, the results shown in Chapter 11 are for experiments performed at least twice and often three times.

Figure 10.13: Median and 95% confidence intervals for the first assay of MDA-MB-231.



On the x-axis is the amount of EGF (ng) added above the Matrigel layer. The y-axis shows the ratio of the sum of fluorescing pixels in the confocal fields at 15, 18 and 21μm divided by the sum of fluorescing pixels at 0, 3 and 6μm.

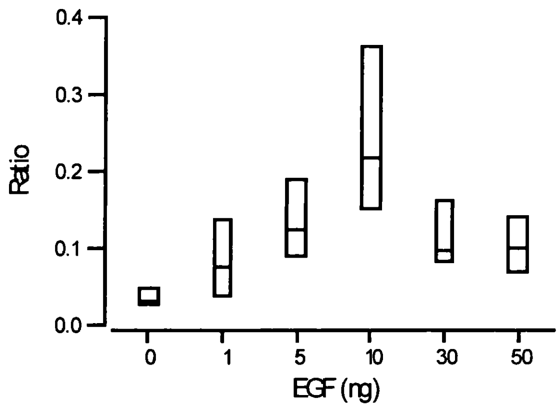
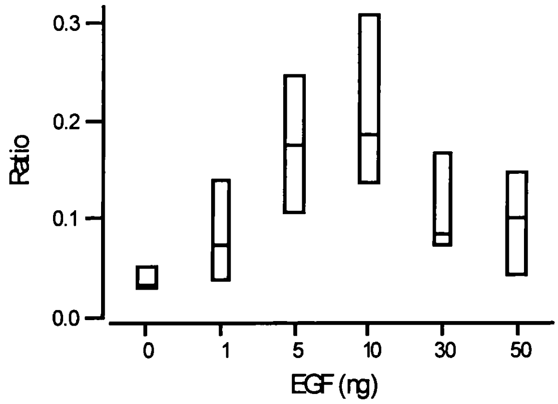


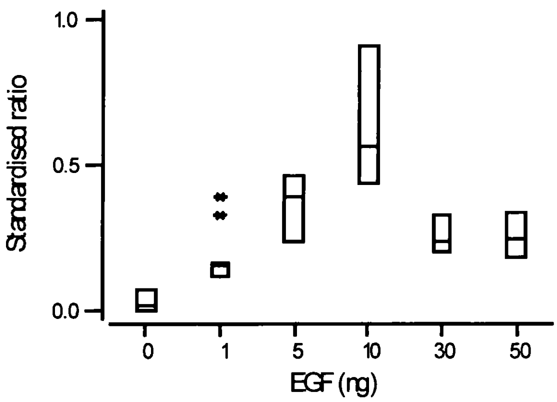
Figure 10.14: Median and 95% confidence intervals for the second assay of MDA-MB-231. The data are presented in the same fashion as above (Figure 10.13).

Figure 10.15: Median and 95% confidence intervals for the third assay of MDA-MB-231.



The data are presented in the same fashion as above (Figure 10.13).

Figure 10.16: 95% confidence intervals for the combined MDA-MB-231 assays.



This assay shows the combined results for the three assays shown above. Results are standardised (facilitating their direct comparison and presentation in a single graphic). The '*' symbol denotes an outlier. The standardised data is also presented in the table below.

EGF (ng)	Readings	mean	Std. Dev.
0	15	0.04	0.046
1	15	0.16	0.094
5	15	0.37	0.164
10	15	0.64	0.255
30	15	0.27	0.104
50	10	0.25	0.106

These results indicated that the assay technique was reproducible and also that the experimental results were reproducible.

Section vi: Invasion assay protocol

All pipettes and containers were kept on ice and the assay protocol was performed in a laminar flow tissue culture hood. Culture media for the assay protocols were supplemented with 1% serum. Since inserts occasionally became infected or the Matrigel dislodged, all assays were run in duplicate.

- Matrigel was removed from -70°C storage and placed in 4°C refrigeration overnight to allow it to liquefy.
- An aliquot was then diluted, 1:1, with culture medium appropriate to the cell line to be studied.
- 80µl of the resultant Matrigel solution was added to the top of the filter in each Transwell insert.
- The Matrigel was then 'gelled' in a humidified incubator at 37°C for one hour.

- Sub-confluent culture flasks of the tumour cell lines to be tested were trypsinised. Cells, re-suspended in culture medium, were counted using a haemocytometer and further diluted, with medium, to produce a suspension of approximately 5×10^5 cells per ml. (Rarely, to achieve the necessary cell concentration, it was necessary to centrifuge the suspended cell solution and remove supernatant.)
- The Transwell inserts were recovered from the incubator, inverted and, to the filter base, was added 100µl of the tumour cell suspension.
- The inserts were returned, in their inverted position, to the incubator for a further 1 hour to allow the cells to adhere to the filter.

- Thereafter, inserts were removed from the incubator and excess cell suspension was aspirated from the filter.
- 750µl of cell culture medium was then added to the wells. Inserts were re-inverted, returning them to their original orientation, and placed in the wells thereby immersing the filter base.
- Quantities of EGF (or other chemoattractant), made up to a 200µl volume with culture medium, were then added above the Matrigel.
- Assays were then returned to the humidified incubator at 37°C, 5% CO_2 for 7 days.

At the end of 7 days assays were fixed.

- Wells of a new 24 well plate were filled with 70% ethanol at room temperature. Test inserts were immersed in these for 20 minutes.
- Inserts were then washed in PBS (phosphate buffered saline) for 5 minutes.
- Cell nuclei were stained by immersing the inserts in a propidium iodide solution (500μg propidium iodide per litre) for 20 minutes.
- This was followed by three further washes of 5 minutes in PBS.
- Finally, inserts were placed in a further 24 well plate immersing the filter in approximately 500μl of PBS. They were then stored in a darkened 4°C refrigerator until they were read.

To prevent contamination, new wells were used at all stages of the fixation process.

Protocol for reading the fixed assay.

- The filter base was wiped to remove cells which had not crossed the filter.
- Filter units were then placed onto a drop of PBS on a glass coverslip on the stage of the BioRad MRC 600 laser scanning confocal microscope. The same scope settings were used for all readings in each of the assays.
- The 20x objective lens and a DM850 filter were selected; this allowed the propidium iodide to be visible as an orange fluorescence.
- Manually, the focus wheel was rotated to take the plane of focus below the filter. Using the computerised focus control, readings were then taken at 2μm steps starting below the filter, until the point of maximum fluorescence was determined. This was the base (0) level for further readings.
- Readings were then taken at 3μm steps, through the Matrigel layer. Starting at the base level and moving upwards, 8 readings, from 0 to 21μm, were taken for each assay.
- The computerised image analysis system was used to determine the number of fluorescing pixels in each confocal section.

Each well was read repeatedly, normally 3-5 times. Between each reading the well was moved slightly, without viewing the field, to try and randomly select fields. To ensure intra-assay standardisation the number of confocal laser scans was always three, the selected level of fluorescence was maintained throughout the assay series and all filter units for a given assay were read at one sitting. Results for repeated assays were standardised as described above.

Interpreting the assay results

1. The sum of pixels at all levels reflected the total number of cells crossing the filter. This value (total pixels) represented a combination of invasion and proliferation.
2. The ratio of cells at higher levels in the Matrigel layer over those at lower levels was also determined (sum of pixels at 15μm+18μm+21μm divided by sum of pixels at 0μm+3μm+6μm). This ratio was considered to indicate the degree of tumour cell invasion induced by the chemoattractant.

Figure 10.17 shows, for a typical assay, the optical sections produced using the invasion assay protocol.

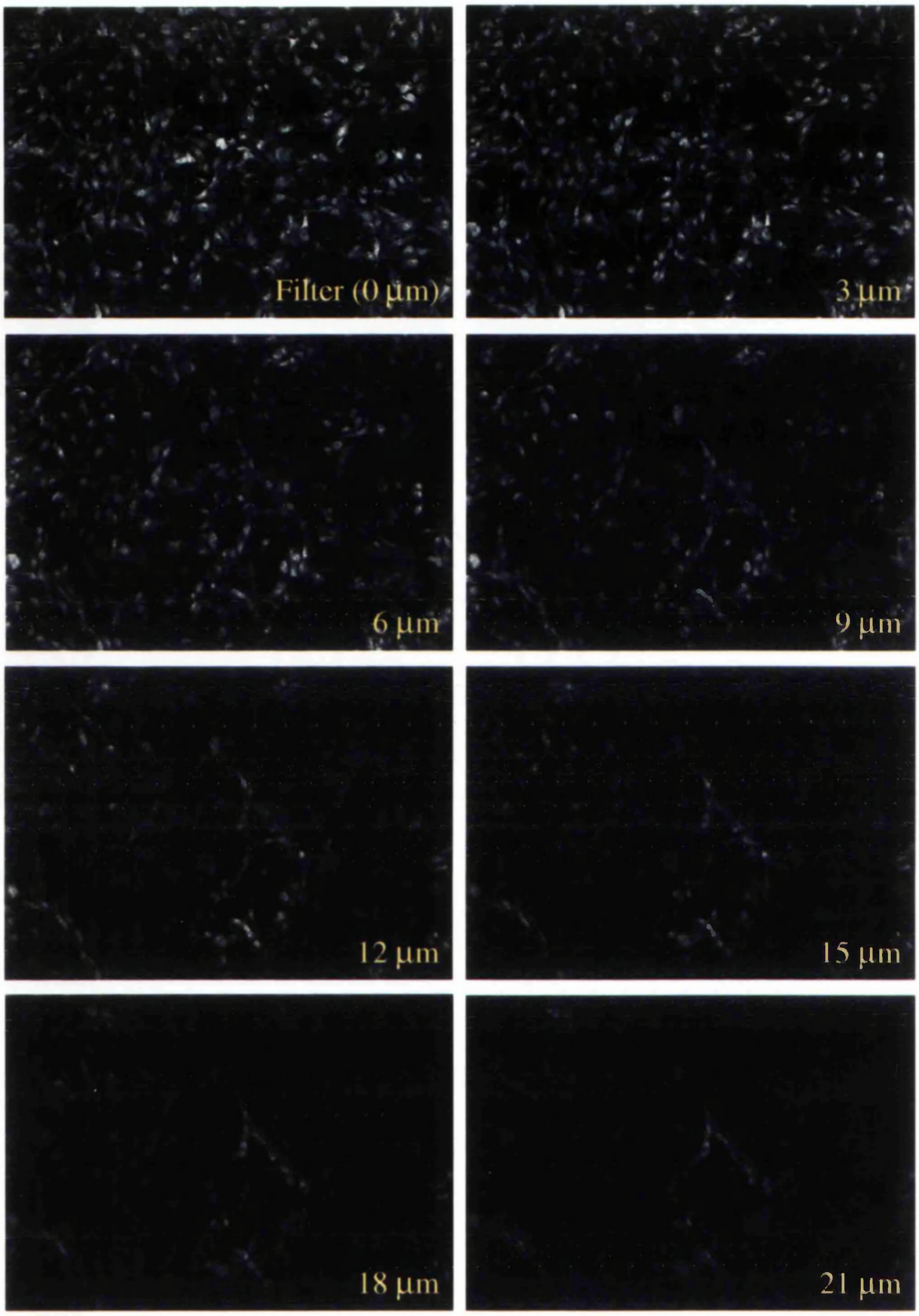


Figure 10.17: Optical sections obtained from an assay of MDA-MB-231 cells using 10ng of EGF as chemoattractant.

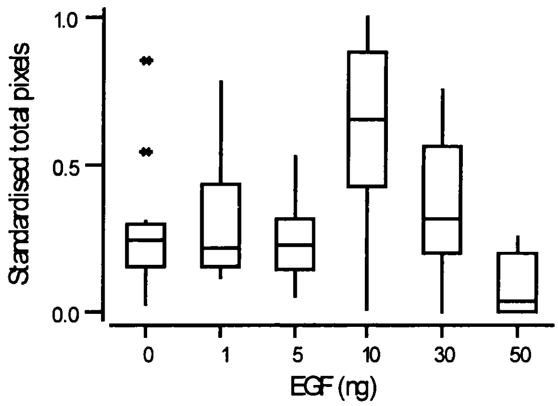
These images were obtained using the BioRad MRC 600 laser scanning confocal microscope, a 20x objective and a DM850 filter. Cells were stained with propidium iodide and sections were taken at 3µm steps, from the level of the filter (0µm) to 21µm. Each image is annotated with its position in the optical stack. Images were computer analysed and stored on optical disc.

Chapter 11:
Results of the invasion studies

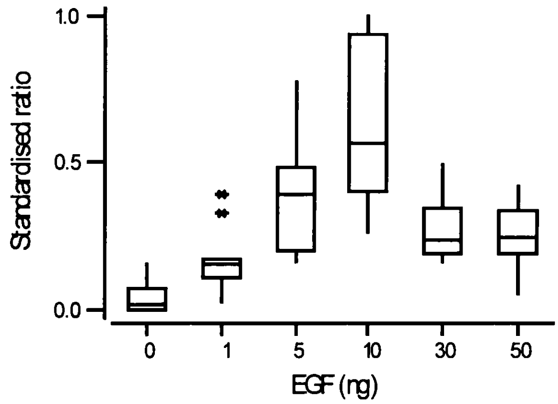
Section i: Using EGF as a chemoattractant

Having established that only certain cell lines would cross the filter, efforts were made to identify maximal invasion for further study. To do this varying concentrations of EGF were used as chemoattractant.

Figure 11.1: EGF stimulated invasion of the MDA-MB-231 cell line.



One way analysis of Variance, $p < 0.0005$



One way analysis of Variance, $p < 0.0005$

The x-axis shows the amount of EGF added above the Matrigel layer. The y-axis in the upper boxplot shows the sum of fluorescing pixels in 8 optical sections above the filter. In the lower boxplot the y-axis shows the ratio of fluorescing pixels in the higher optical sections over those in lower optical sections. Both boxplots show medians and interquartile ranges. The plot shown is derived from standardised data from 3 experiments. The '*' symbol denotes an outlying result.

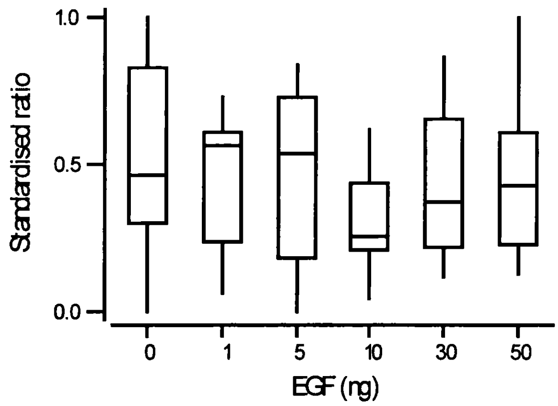
Interpretation

Both boxplots demonstrate a dose-response curve; more invasion was produced by increasing amounts of EGF up to approximately 10ng, higher levels stimulated less invasion. This observation was more apparent in the second boxplot. This may result from an EGF concentration gradient in the Matrigel layer; increased EGF added above the filter generating an increased gradient and greater invasion of those cells that crossed the filter. The reduced invasion observed with higher concentrations may result from cells succumbing to toxic levels of EGF. This dose-response profile, with cells responding to lower EGF concentrations but failing to do so to higher concentrations, has been reported in studies of tumour cell proliferation (Osborne 1980).

Maximal invasion was produced by adding 10ng of EGF above the filter. This amount was used in subsequent assays in which modulation of invasion was studied.

Figure 11.2: EGF stimulated invasion of the BT20 cell line.

One way analysis of Variance, $p < 0.0005$



One way analysis of Variance, $p = 0.774$

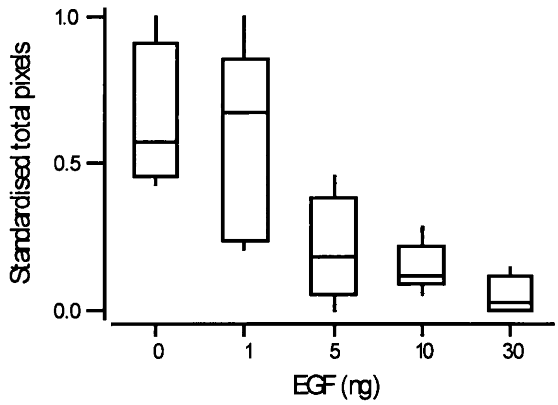
Figure 11.2 (continued): EGF stimulated invasion of the BT20 cell line.

The results shown here are for the BT20 cell line (2 experiments). They are presented in the same manner as those, above, for the MDA-MB-231 cell line (Figure 11.1).

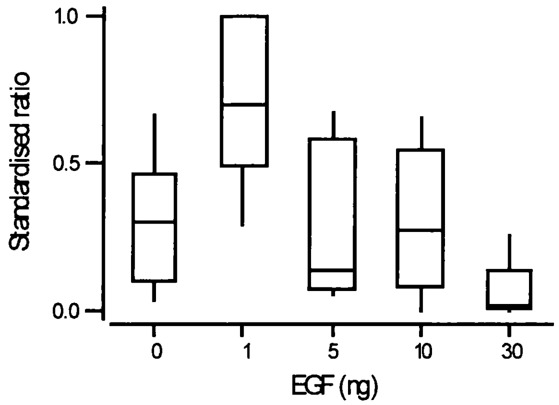
Interpretation

The upper boxplot demonstrates a clear dose response curve with 1-5ng of EGF provoking the greatest invasive response. The lower boxplot is more difficult to interpret; there is no obvious difference between the groups, this may be due to the relatively low magnitude of invasion induced by any of the EGF concentrations.

Figure 11.3: EGF stimulated invasion of the MCF7ADR cell line.



One way analysis of Variance, $p < 0.0005$



One way analysis of Variance, p = 0.001

Figure 11.3 (continued): EGF stimulated invasion of the MCF7ADR cell line.

These results are for the MCF7ADR cell line (2 experiments). They are presented in the same manner as those, above, for the MDA-MB-231 cell line (Figure 11.1).

Interpretation

Again there was relatively little invasion in this assay series making the results more difficult to interpret than for the MDA-MB-231 cell line. The upper boxplot shows relatively few cells crossed the filter; in the control assay and that with 1ng of EGF there was invasion, but when further EGF was added fewer cells crossed the filter. The results shown in the lower boxplot suggested that, compared with the control, addition of 1ng of EGF stimulated invasion further into the Matrigel layer.

Summary

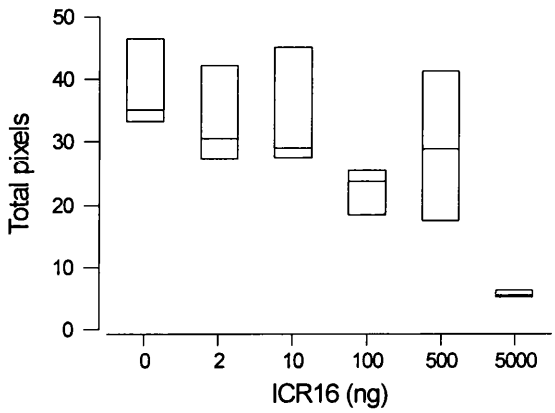
All three cell lines produced an invasive response which was modulated by EGF. The magnitude of this response varied with the cell line, but in all cases addition of EGF increased invasion. Invasion was most evident with MDA-MB-231 cells and these were used for subsequent experiments.

Section ii: Antibody experiments

The aim of these experiments was to determine if invasion was mediated via EGF receptor. If invasion could be abrogated using monoclonal antibody directed at, and inhibitory to, EGF receptor then this would support the contention that the invasive response was the result of EGF stimulation of EGF receptor.

ICR9 and ICR16 antibodies were used (a kind gift from Dr C Dean, ICR Sutton). Both are rat monoclonal antibodies of the IgG class, they bind a 170kD protein in Western blots and in Scatchard analysis they compete with EGF to bind EGF receptor (Modjtahedi 1992). Modjtahedi *et al* described 8 antibodies (Modjtahedi 1992), of these ICR16 demonstrated greatest inhibition of EGF binding and ICR9 enhanced ligand binding. They also reported the effects these molecules had on cellular proliferation. ICR9 stimulated proliferation, most efficaciously in cell lines expressing low levels of EGF receptor. ICR16 reduced proliferation.

Figure 11.4: Adding ICR16 above the Matrigel layer.



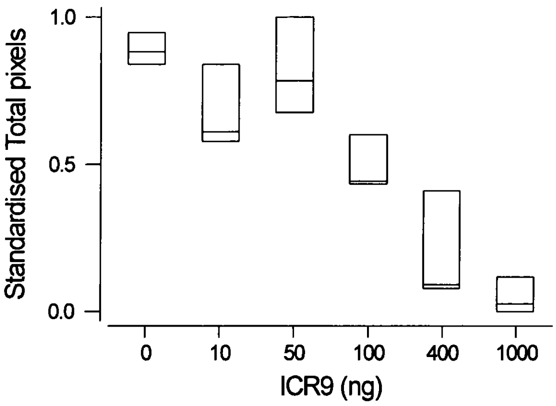
One way analysis of Variance,

p = 0.289

The y-axis reports the sum of pixels in a stack of 8 optical sections. The x-axis, the amount of ICR16 added above the Matrigel. The plots are of the median and interquartile ranges. Analysis of Variance is reported (excluding the 5000ng group).

Adding 5000ng ICR16 reduced the number of cells that crossed the filter, but there were few cells in the well below the filter and these appeared poorly viable. This was interpreted as a non-specific toxic effect of this concentration of antibody. For this reason, those assays to which 5000ng were added were excluded form the statistical analysis, The results demonstrated that antibody did not reduce invasion (Analysis of variance p=0.289). This was not the expected result and raised the possibility that the antibody, a molecule very much larger than EGF, might have been unable to diffuse through the Matrigel layer. The experiments were, therefore, repeated, this time adding antibody to the lower chamber. Results are shown below.

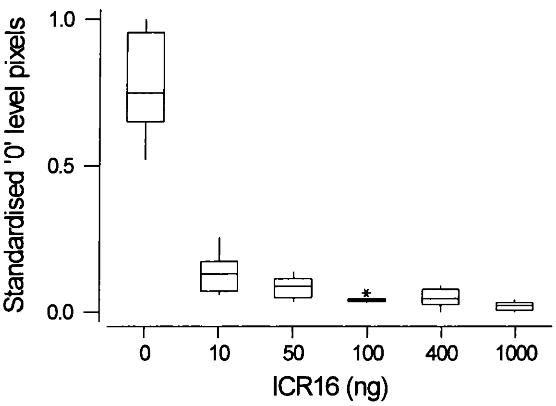
Figure 11.5: Adding antibody below the Matrigel layer.



One way analysis of

Variance,

$p < 0.0005$



One way analysis of

Variance,

$p < 0.0005$

The upper boxplot is for the antibody ICR9 and the lower plot for ICR16. For both, the x-axis shows the amount of antibody (ng) added to the assay well. The y-axis in the ICR9 plot shows the median and interquartile ranges of the sum of pixels in the 8 optical sections, 0-21μm, above the Matrigel layer. In the ICR16 plot the data depicted is the median and interquartile ranges of the pixels at the filter, 0μm, level only. Standardisation of results allowed data from 3 experiments to be shown. Outlying results are indicated by the '*' symbol.

The ICR9 plot shows that invasion was minimally effected by low concentrations of this antibody, but at higher concentrations there appears to be reduced invasion. Modjtahedi, presenting data for three cell lines, had reported that ICR9 increased EGF binding of the EGF receptor (Modjtahedi 1992). His results for both HN5 and A431 cell lines suggested that higher concentrations of antibody further increased ligand binding of receptor. Addition of ICR9 might, therefore, have been expected to increase invasion in our studies. However, Modjahedi also reported that for EJ cells, those with fewest receptors and most reflective of levels in breast cancer, increased antibody concentration increased EGF binding only to a point, after which further increases reduced ligand binding. This could account for the reduced invasion seen at higher ICR9 concentrations (Figure 11.5). An alternative explanation is that Modjtahedi's fibroblast experiments were not augmented with EGF and, as he reported, ICR9 stimulated less proliferation than EGF alone. Our invasion studies were augmented with EGF, and the invasion so stimulated may have exceeded that that could be induced by ICR9.

Adding ICR16 antibody to the assay well produced very little invasive response and, therefore, the boxplot for this antibody shows the results of the pixel counts at the filter (0) level, rather than, as previously, the sum of pixels in 8 optical sections above the filter. To allow direct comparison of results for both antibodies, the ICR9 data was analysed in a similar manner. Plots of the median pixel counts for each antibody are superimposed below.

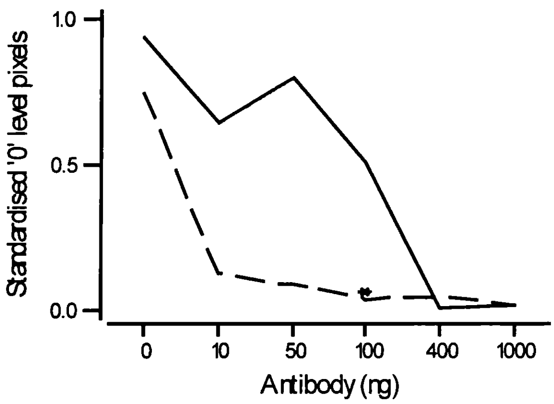


Figure 11.6: Plot of the median standardised pixel counts for the ICR antibodies.
The continuous line represents ICR9, the broken line ICR16. On the x-axis is the amount of antibody added to the assay well. On the y-axis is the median pixel count at the filter, 0μ m, level. To allow the plots to be superimposed the results are standardised (data from 3 experiments). The '*' symbol denotes an outlier.

Interpretation

Demonstrated clearly was that both antibodies reduced invasion, and that this effect was more pronounced at lower concentrations with the ICR16 antibody. The ICR9 control produced a differential reduction in invasion which inferred the former was not a non-specific toxic effect of the IgG class of antibody. Note that the x-axis is non-linear and that reduction in invasion produced by ICR16, compared to ICR9, is more pronounced than is immediately evident.

Summary

These experiments demonstrated that EGF induced invasion could be abrogated using monoclonal antibody directed to EGF receptor, and thus that this receptor, at least in part, modulated the invasive response.

Section iii: Tyrosine kinase inhibitor experiments

The majority of tyrosine kinase inhibitors function by competing with the enzyme's ATP binding site (reviewed in Levitski 1995) and it has proved possible to produce inhibitors specific to individual tyrosine kinases, including that of EGF receptor. Since higher levels of EGF receptor expression are associated with poor prognosis in many human cancers, there has been considerable interest in the therapeutic potential of this group of drugs (Buchdunger 1994, Zhang 1995, Shawver 1994). The aim of these experiments was to establish that tumour cell invasion could be obviated by targeting the intra-cellular signalling of the EGF receptor.

The tyrosine kinase inhibitor used was 4,5-Dianilinophthalimide (DAPH). This agent has been shown to selectively inhibit ligand induced EGF receptor autophosphorylation (Buchdunger 1994), and was a kind gift from Dr Nicholas Lydon (CIBA-Geigy, Basel, Switzerland). Experiments were conducted in a manner similar to those for the antibodies. DAPH was placed above the Matrigel; DAPH molecules are very much smaller (Molecular weight of 337, Buchdunger 1994) than the large antibody molecules (approximately 1300 amino acids, Alberts 1989) and there was, therefore, not the same concern that they might fail to diffuse through the Matrigel. MDA-MB-231 cells were used for these studies.

DAPH was supplied as a 10mM solution in Dimethyl sulphoxide (DMSO). To ascertain the effect of this solvent an experiment was conducted growing MDA-MB-231 cells in varying concentrations of DMSO. Aliquots of a cell suspension were incubated in RPMI augmented with 10% foetal calf serum and DMSO. After one week cells were trypsinised, re-suspended in a uniform volume and counted on a haemocytometer. Prior to trypsinisation cells were viewed with a light microscope. Results are shown in Table 11.1.

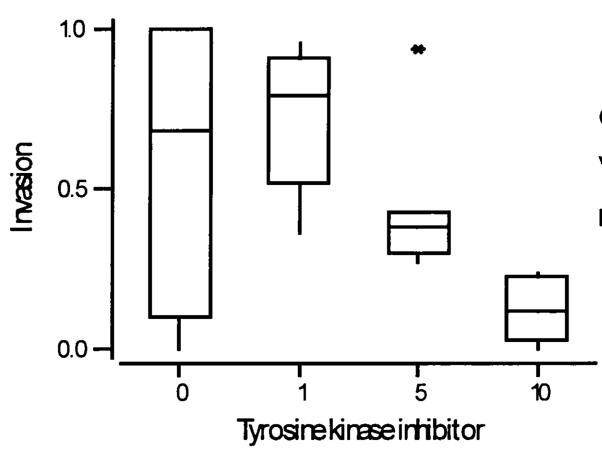
Concentration of DMSO (%)	Haemocytometer counts (mean)	Light microscopy
0	14.4	normal cell appearance, confluent
0.25	14.0	normal cell appearance, confluent
0.5	13.4	normal cell appearance, confluent
1	16.0	normal cell appearance, confluent
2	13.0	cells appeared less healthy and were sub-confluent

Table 11.1 : Determining the significance of DMSO on MDA-MB-231 cell proliferation.

This experiment indicated that cells proliferated at a normal rate in concentrations of up to 2% DMSO, (Analysis of Variance for cell counts, $p = 0.127$). At higher concentrations, light microscopy revealed cells appeared less healthy. Since the effect of DMSO on invasion was unknown, its concentration was kept constant in the subsequent invasion studies. DMSO was added to the aliquots containing DAPH to produce a final concentration of 0.5% DMSO (v/v).

The invasion assay was repeated varying the concentration of tyrosine kinase inhibitor added above the Matrigel (Figure 11.17). No invasion was recorded at the 50 and 100μM concentrations of inhibitor which are therefore not plotted.

Figure 11.7: Using tyrosine kinase inhibitor in the invasion assay



One way analysis of Variance, $p < 0.0005$

The boxplot shows the medians and interquartile ranges. The x-axis shows the concentration of tyrosine kinase inhibitor (μM) in the 200μl aliquot added above the Matrigel layer. The y-axis shows invasion as the standardised (2 experiments) total fluorescing pixels. The '*' symbol denotes an outlier.

Interpretation

In the antibody experiments ICR9 functioned as a control molecule and was used to show that reduced invasion seen with low levels of ICR16 was not due to a non specific toxic effect. No such simple control was available for the tyrosine kinase inhibitor experiment. The first such experiment indicated that at concentrations of 50μM and 100μM, no cells crossed the filter. This might have resulted from a non-specific toxic effect and, to address this possibility, filters from the second assay were studied before the 'bottom wipe'. Fluorescent and light microscopic examination of the filters was performed. The assay wells were also assessed.

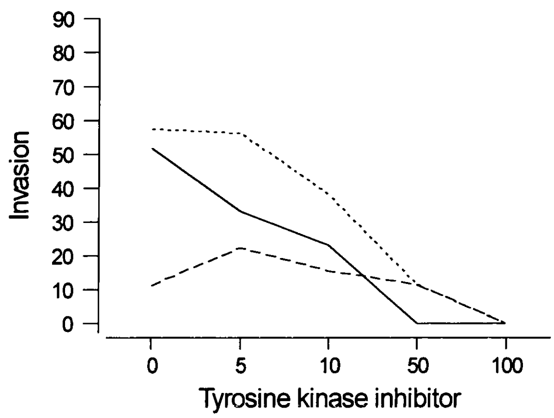
Inhibitor (μM)	CELLS IN THE ASSAY WELLS	APPEARANCE AT THE FILTER
0	Moderate numbers of cells which were bipolar	Moderate numbers of cells
5	Moderate numbers of cells which were bipolar	Possibly more cells than at 0μM inhibitor
10	Moderate numbers of cells which were bipolar	Moderate numbers of cells
50	Reduced numbers of cells which were rounded	Reduced numbers of cells
100	Very few cells	Virtually no cells

Table 11.2: Appearance of the cells in the tyrosine kinase inhibitor experiments.

At concentrations of 50μM, or above, the tyrosine kinase inhibitor significantly reduced the number of cells growing in the assay. This may have been either through its function as a tyrosine kinase inhibitor or via a direct toxic effect. Tyrosine kinase inhibitor concentrations up to 10μM did not appear to significantly reduce the numbers of cells in the assay.

In an effort to quantify these observations readings were taken from the unwiped filter base. The usual protocol was used to identify maximum fluorescence which was considered to reflect the number of cells growing on the underside of the filter. This method had the advantage that it was simple and that it maintained the integrity of the filter/Matrigel assembly which could then be studied in the usual fashion. There were, however, a number of reservations. As indicated above (Figure 10.7), determining the level of the filter, without wiping its base, was difficult. Additionally, cells showed some propensity to grow down from the filter in clumps (evident using light microscopy) and cells in these clumps might not be included in the thin optical section that included the filter. Further, no measurement of the cells in the assay well was available. Despite these reservations the measurements were considered to provide a relative, if crude, estimate of the number of cells growing at the filter. The results are shown below.

Figure 11.8: Invasion and proliferation in the tyrosine kinase inhibitor experiments



The plot shows, on the y-axis, the median pixel count of three assays and on the x-axis, the concentration of tyrosine kinase inhibitor (μM) in the 200μl aliquot added above the Matrigel layer. Three lines are plotted. The solid line depicts the sum of pixels above the filter (a measure of invasion), determined after wiping the filter. The broken line is the pixel count below the unwiped filter. The dotted line is the sum of pixels above and below the filter (an estimate of the total number of cells in the assay). (These data are from one experiment).

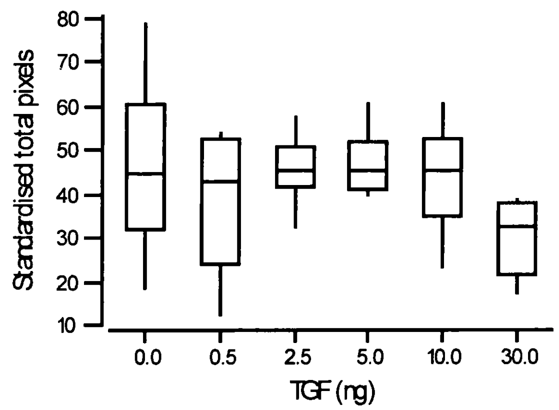
Summary

No cells crossed the filter at Tyrosine kinase inhibitor concentrations of 50μM or greater. At lower concentrations, up to approximately 5μM, the inhibitor reduced the number of cells invading the Matrigel layer but did not appear to effect cellular proliferation. These results indicated that EGF receptor specific tyrosine kinase inhibitor could reduce breast tumour cell invasion. The inhibitor, used in the experiments above, has been shown to produce potent *in-vivo* anti-tumour activity (Burchdunger 1994). This was measured as a reduction in volume of implanted tumour xenografts. Our studies suggest that reduced tumour cell invasion may, at least partly, explain any clinical benefit from these drugs.

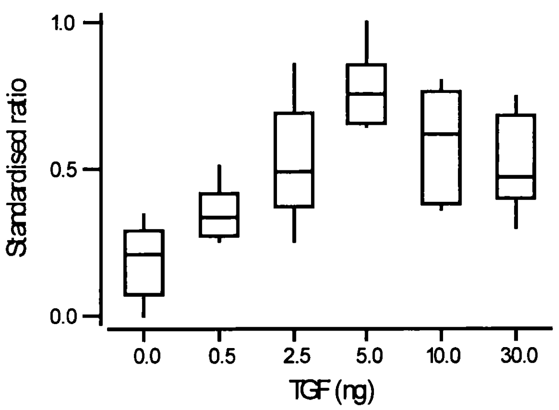
Section iv: Alternative chemoattractants

The assay protocol was developed chiefly to analyse the effect of EGF on EGF receptor, however, it was possible to study invasion induced by other attractants.

In-vitro studies provide evidence that Transforming Growth Factor Beta (TGF-β) increases cellular proteolytic activity and increases metastatic potential (Welsh 1990). At a cellular level, TGF-β from MCF7 cells induces fibroblasts to produce the stromal protein, tenascin, this in turn causes breast tumour cells to lose cell to cell, and cell to substrate, contacts (Chiquet-Ehrismann 1989). In the clinical setting, breast tumours expressing higher levels of TGF-β have a higher incidence of nodal metastases (Walker 1992). Thus TGF-β may have a role in the process of invasion and the assay was repeated substituting TGF-β for EGF.



One way analysis of Variance, $p = 0.229$



One way analysis of Variance, $p < 0.0005$

Figure 11.9: Invasion induced by TGF-β

MDA-MB-231 cells were used for these (2) experiments. Results are presented in a similar fashion to those in Figure 11.1

Interpretation.

The upper boxplot (Figure 11.9) shows no obvious difference in the numbers of cells that crossed the filter, however, analysis of the ratio, indicated that TGF-β stimulated cells that did cross the filter to invade into the Matrigel layer. These results, using TGF-β as chemoattractant supported the hypothesis that this molecule does promote tumour cell invasion. They also demonstrated that the assay protocol was suitable for assessing different chemoattractants.

Additionally, these results can be used to support the proposed methods of assay interpretation. The MD-MB-231 cell line is oestrogen receptor negative and TGF-β has been shown to inhibit the proliferation of oestrogen receptor negative cell lines (Arteaga 1988) but, as indicated above, it may promote tumour cell invasion. In this assay, the upper boxplot shows increasing TGF-β concentrations had minimal effect on the number of cells above the filter, with a trend to reduction at the 30ng concentration. However, the ratio shown in the lower boxplot suggests this molecule could induce invasion.

Chapter 12:
Discussion; invasion studies

Section i: Establishing the invasion assay

Epithelial cells require intact basement membrane for anchorage and growth (Kleinman 1981, Wicha 1980), and in invasive carcinoma the normality of both of these cell attributes is characteristically lost, as is the integrity of the basement membrane layer (Liotta 1984). Scanning electron microscope images of carcinoma in-situ reveal focal defects in the basement membrane and this may represent the earliest stages of progression to invasive carcinoma (Liotta 1986). These observations have encouraged cancer scientists to explore the relationship between cancer cells and basement membrane and specifically the former's invasion of the latter.

Appropriate invasion models of human cancers, and particularly breast cancer with its protracted clinical course, have been difficult to develop. The ideal invasion model would:

- allow study of specific phenotypic conversions observed in human carcinoma *in-vivo*,
- take into account the complex interactions between cells and micro-environment,
- allow easy experimental manipulation of genetic information and micro-environment.

At present no such assay exists.

Animal models fail to satisfactorily reflect human disease. Of the *in-vitro* techniques, the biological invasion assays, which might most accurately reflect *in-vivo* events, are poorly reproducible. Thus, despite some concern that they might poorly reflect the behaviour of human carcinoma *in-vivo* (Noel 1991), reconstituted basement membrane methodologies have provided much of our understanding of the genotypic, and phenotypic, changes that result in invasive cancer.

A large number of reconstituted basement membrane assays have been described and this probably reflects the fact that none is ideal. The assay protocol, described in this work, is of novel design and addresses some of the difficulties encountered with previous methodologies. The protocol was developed under supervision from Dr B Ozanne at the Beatson Institute for Cancer Research (Hennigan 1994). Its principle differences, compared to existing protocols, relate to the depth of the Matrigel layer and the technique used to measure invasion.

Previous reports describe the use of a thin Matrigel layer, containing relatively little Matrigel protein. In these assays invasion has been measured as numbers of cells crossing the filter, or has been defined in terms of Matrigel degradation. Neither technique allows study of cells actually invading the basement membrane barrier. Our protocol, by incorporating a thick Matrigel layer, offered the opportunity to study the progress of tumour cells as they passed through this barrier. To be realised, this advantage required that invasion be measured within an intact Matrigel layer. This problem was overcome by the use of confocal microscopy. Coupled to computer assisted image analysis, confocal scans provided an estimate of the number of cells in progressive, thin optical sections through the Matrigel layer. This method of measuring invasion had not previously been reported.

Using confocal microscopy allowed estimation of invasion at higher levels, compared to that at lower levels, in the Matrigel. This ratio, as opposed to the total number of cells at all levels above the filter, provided evidence that the assay did measure invasion and did not simply reflect proliferation. Thus, our results suggested that EGF receptor stimulation mediated an invasive response that was independent of cellular proliferation. This corroborated published work comparing invasion of wild type and mitogenically active but motility deficient EGF receptor (Xie 1995). Subsequently, in the same lab and using a similar invasion assay, Brunton demonstrated that EGF can be used to promote invasion of a colorectal cancer cell line. This second study used both [^{3}H] thymidine incorporation and tetrazolium dye reduction assays to demonstrate that invasion was independent of proliferation (Brunton 1997).

A further fundamental variation in this assay system, as opposed to those previously reported, was the orientation of the assay components; cells, filter and Matrigel. In conventional assays cancer cells have been seeded above the Matrigel layer, which coats the upper surface of the filter. In the protocol used in this series of experiments, Matrigel was added above the filter and cells were seeded on the filter under-surface. By seeding them below the filter, cells invading the Matrigel had to pass through 8μm pores. This required them to do so as individual cells and may have allowed better appreciation of their invasion than when cells were plated above the Matrigel, with which they interact forming multicellular aggregates, resembling in structure, benign breast (Streuli 1991, Darcy 1991). For those cell lines that did invade, fewer than the $5x10^4$ cells seeded onto the filter under-surface will have adhered to it and during the week long incubation only some of these entered the Matrigel layer. The proportion that did so could not be determined accurately since, after incubation, cells above the filter were likely to reflect invasion and proliferation; cells within the Matrigel seemed contiguous (Figure 10.17). In fact, *in-vivo* invasion by breast cancer is likely to reflect a combination of these attributes, both of which characterise carcinoma cells.

Another prime advantage of reconstituted basement membrane assays is that they generate results that are reproducible (reviewed in Hendrix 1989). Although the confocal technique for measuring invasion was relatively complex and required special equipment, our data showed that it was reproducible. In addition the use of a thick Matrigel layer circumvents concerns regarding the integrity of the Matrigel barrier and may have improved the reproducibility of this technique over those employing a thin layer.

Relatively few breast tumour cell lines produced an invasive response that was detectable using our assay; only three of nine cell lines. Whilst this finding was disappointing, it was not entirely unexpected. It is notoriously difficult to culture malignant cells from primary breast tumours (Wolman 1985) and many of the commonly studied breast tumour cell lines are poorly representative of cells found in the breast carcinomas of patients (reviewed in Taylor-Papadimitriou 1993). The initial hope, that this assay might be applied to primary breast cancers, providing an insight into their likely clinical behaviour, proved to be unrealistic. This stated, the assay was reproducible and proved relatively robust, allowing study of various chemoattractants. It was also easily modified to study manipulation of the invasive response.

Section ii: Results of the invasion assay

Up-regulated EGF receptor signalling has been correlated with tumour progression in human neoplasia, however, the cell behaviour which is promoted remains undefined. There is considerable evidence that migration and invasion of malignant cells may be partly dependent on local chemotactic factors (Terranova 1986, Hujanen 1985). Similarly there is good evidence that breast tumours with cells expressing higher levels of EGF receptor (reviewed in Klijn 1992) and tumours with high EGF content (Kamameris 1993) are associated with poor prognosis. The purpose of this series of experiments was to explore the possibility that EGF binding of EGF receptor might convey an increased invasive potential on breast cancer cells.

In Chapter 4 (Breast tumour cell invasion and EGF receptor) a number of references are cited that indirectly support the contention that elevated levels of EGF receptor correlate with increased invasion in a number of malignancies. There are, however, relatively few reports directly linking up-regulated EGF receptor signalling to increased tumour cell invasiveness. The experiments, presented in this work, indicated that EGF interaction with the EGF receptor induced invasion of human breast cancer cells. Blocking this response with both receptor directed antibody and tyrosine kinase inhibitor demonstrated that invasion was mediated via the EGF receptor.

The MDA-MB-231 cell line was stimulated to invade more than the others, but invasion was also seen with the BT20 and MCF7 Adriamycin resistant lines. Although few tumour cell lines invaded, those that did expressed higher levels of EGF receptor (see Chapter 6, section iv). This concords with recent reports that EGF receptor expression directly correlates with metastatic potential in colorectal carcinoma (Radinsky 1995). However, the relationship, between receptor expression and invasion, was not absolute and the degree of invasion was not proportional to the level of receptor expression. Thus it would appear that, whilst EGF receptor stimulation has a role in tumour cell invasion, there are other factors which are important.

The cellular morphology of the MDA-MB-231 line was unlike those of the other lines studied (Table 10.4). It has been suggested that breast cancer cells undergo an 'epithelial to mesenchymal transition' and in doing so become more invasive (Bae 1993); this may explain the high level of invasion seen with these cells which are elongate with pseudopods.

Mesenchymal morphology, in breast cancer cells, has been associated with oestrogen receptor negative/vimentin positive phenotype (Sommers, 1989). Vimentin is an intermediate filament glycoprotein which is important in cell morphology and motility. Elevated expression of vimentin in breast cancer cells has been associated with increased tumour cell invasiveness (Thompson 1992, Bae 1993) and poor prognosis (Domagala 1990). In turn, elevated EGF receptor expression has been associated with vimentin expression. Syllogistically, EGF receptor signalling may influence cell motility, possibly via an action on vimentin. Supporting this hypothesis is the observation that mesothelial cells, stimulated to rapid growth by EGF, increase vimentin expression and decrease cytokeratin expression (Connell 1983).

Brunton's group reported a model for the adenoma to carcinoma sequence in colorectal disease (Brunton 1997). A corollary for breast carcinoma is provided by studies of MCF7 and MCF7ADR lines. The MCF7ADR line, derived from MCF7 cells by incubation with increasing concentrations of adriamycin (Vickers 1988), has gained vimentin expression and lost oestrogen receptor expression (Thompson 1992), thereby, gaining an invasive phenotype (Bae 1993) and an elevated level of EGF receptor expression. This concurs with the experimental results reported above; the MCF7 line failed to invade the Matrigel layer but the adriamycin resistant derivative, expressing eight times as much EGF receptor (Vickers 1988), produced a dose related invasive response to EGF. It appears, therefore, that EGF receptor stimulation may influence invasion, and that this influence may, in part, be via an action on vimentin expression. However, attributing EGF promoted invasion solely to an effect on vimentin expression is not justified.

For example, Brunton's group also demonstrated that EGF induced an increase of c-Src activity, relocation of the c-Src to focal adhesions and phosphorylation of focal adhesion kinase (FAK) in invading carcinoma cells. This did not occur in the non-invading adenoma cell line. FAK is a tyrosine phosphorylated protein, localised to adhesions; discrete cellular regions where intergins interact with extracellular proteins. Fibroblast studies indicate that FAK deficient cells have reduced motility (Ilic 1995), in epithelial cells FAK phosphorylation and expression correlates with increased cell motility (Akasaka 1995, Matsumoto 1994). Furthermore increased expression of FAK is associated with invasive tumours of breast (and colon) (Owens 1995).

There is also evidence to suggest that EGF receptor signalling may have an effect on cadherin mediated cell to cell adhesion (Shiozki,1995). EGF stimulation of TE-2R oesophageal cancer cells resulted, not in down-regulated expression of E-cadherin, but in its relocation within the cell. It would appear, therefore, that EGF receptor stimulation influences tumour cell invasion via a number of complex intracellular signals which result, not only in altered expression, but also in relocation of cellular proteins. The precise signalling interactions remain far from clear.

Since expression of the EGF receptor correlates with poorer prognosis, this receptor represents an obvious target for therapeutic intervention. A number of studies have explored the use of antibodies targeted at EGF receptor to reduce growth of cancer cells *in-vitro* and *in-vivo* (reviewed in Modjtahedi 1994). Antibodies can effect this action by blocking ligand binding, thereby reducing receptor signalling, or, *in-vivo*, by stimulating the host immune system, in which event they are directing the host system to EGF receptor bearing cells. The most efficacious antibodies function in both ways (Modjtahedi 1993).

The invasion assay could only address the effect of reduced receptor signalling, but the antibody experiments clearly demonstrated that tumour cell invasion could be reduced by monoclonal antibody directed against, and inhibitory to, the receptor. Clinical use of immunotherapy has focused on the use of combinations of antibody and cytotoxic agents. The use of these compounds is, however, limited by difficulties using mouse or rat antibodies to which the host produces an immune reaction. Although this may initially direct the host immune system to tumour cells it can limit the effectiveness of repeated treatments by inducing anaphylaxsis (Modjtahedi 1994). Whilst these difficulties may be overcome by using humanised antibodies or genetically engineered antibody fragments there are other problems with the use of larger combined molecules.

EGF receptor expressed by most cancer cells, including breast cancer cells, appears to be of wild type. Selective targeting of cancer cells is therefore difficult. This problem is compounded by the fact that hepatocyte borne EGF receptors can clear large quantities of circulating EGF (Yanai 1990). Thus any targeting reagent must extravasate rapidly to minimise liver clearance. This criterion is met by low molecular weight molecules, such as the tyrosine kinase inhibitors. These small molecules also overcome a further problem for targeted therapies, that of penetration of solid tumours. Tyrosine kinase inhibitors are therefore generating considerable clinical interest (reviewed in Davies 1996).

Interpretation of signal transduction blockade is complicated by the web of interactions arising from receptor activation, but, in this regard, the tyrosine kinase inhibitors have the advantage that they block the initiating intracellular signal. Receptor blockade also produces the potential for lateral signal transduction and, thereby, unexpected *in-vivo* results. This stated, experiments using DAPH showed that tumour cell invasion could be reduced significantly by relatively small drug concentrations. Therefore, this group of drugs may have potential, both to reduce metastasis from established cancers, and as prophylaxis in patients at high risk of developing breast carcinoma.

Radioimmunohistochemical studies, of both primary breast cancers and normal breast tissues indicated that most breast cancers express very much lower levels of EGF receptor than does normal breast (Figure 6.1). This concurs with other reports (Dittadi 1993) and, in conjunction, with *in-vitro* evidence that the efficacy of EGF-bound toxins was directly related to receptor levels (Kirk 1994), it might appear that the EGF receptor is a poor therapeutic target in breast cancer. However, animal studies of EGF receptor directed toxins are encouraging (Pai 1991) and there is also evidence that cancer cells with fewer EGF binding sites, may be more susceptible to therapeutic targeting than normal cells (Kameyama 1994). The latter provides the potential advantage of a reduced therapeutic dose and reduced systemic side effects.

Whilst accepting that tumour cell invasion is the culmination of a large number of cellular processes, the data presented in this section does suggest that EGF receptor signalling has a role in breast cancer cell invasion. This may be the mechanism of the prognostic significance of higher levels of EGF receptor expression in breast cancer and abrogation of this invasive response would appear to be an desirable therapeutic goal.

GENERAL DISCUSSION

The 'specific aims' of the thesis were, to a greater or lesser extent, fulfilled:

- EGF receptor was quantified in a large series of primary breast cancers.
- It was possible to demonstrate the superiority of the radioimmunohistochemical method over more conventional techniques.
- The accuracy of this method did allow observations that could not have otherwise been determined.
- It was also possible to demonstrate that receptor might influence breast tumour cell invasion.

Yet, the role of EGF receptor in breast tumour cell biology remained elusive. This requires that we consider the value of accurate measurement of factors, such as EGF receptor, in clinical specimens.

Was it reasonable to assume that more accurate receptor estimation would translate to clinical benefit?

Higher levels of receptor expression did predict poorer outcome but the clinical value of this observation is questionable given that more established, more easily obtained, prognostic indicators are much better predictors of outcome. Clearly, outcome is dependent on a host of biological characteristics both of the tumour and patient. Assessment of any feature in isolation is akin to trying to deduce the outcome of a football match by watching a single player. For the type I tyrosine kinase receptors this analogy may be particularly valid since we know that members of this receptor family interact functionally and, radioimmunohistochemistry suggests, that their expression is interdependent. Further, within a tumour there will be a number of genotypically distinct clones. Sampled tissue is, therefore, not necessarily representative of the tumour or indicative of its clinical course. Equally, assessment of the whole tumour mass, not withstanding its impracticality, is unlikely to be fulfilling since prognosis will be determined by 'aggressive' genotypes that may be poorly represented in the tumour as a whole. Although this appears discouraging, it should not detract from efforts to measure biological markers more accurately. After all, it is probable that any clinical advantage gained from these markers will not pertain to their measurement *per se*, but rather to an improved understanding of tumour biology.

Did more accurate receptor measurement improve our understanding of breast cancer?

In recent years, there has been little improvement in the prognosis of those patients diagnosed with breast cancer. We now accept that this disease is the culmination of a series of genetic events and these will need to be unravelled if treatments are to become more sophisticated. This, in turn, will require more accurate assessment of the cellular changes that occur in cancer. Limitations in measurement methods, together with a perceived lack of value of more accurate quantification, has caused many biological markers to be categorised, positive or negative. This is clearly artificial as most biological characteristics are subject to a range of values. This is no less true of the EGF receptor than of other biological characteristics and more accurate receptor measurement certainly provided our most interesting results;

- a direct relationship between tumour size, in millimetres, and EGF receptor expression,
- an inverse relationship between expression levels of EGF receptor and oestrogen receptor,
- and, although not specifically reported in this work, an inverse relationship between EGF receptor and c-*erb*B-2 encoded receptor, in those tumours without c-*erb*B-2 gene amplification (Robertson 1996).

Inter-relationships such as these offer new avenues for further study. Their identification may begin to allow us to piece together the molecular signalling that defines the behaviour of tumour cells. They also offer therapeutic potential, if drug induced modification of one factor can militate another. These observations were achieved only by analysis of EGF receptor expression as a continuum. This, in turn, was only possible because of the accuracy of the radioimmunohistochemical method.

How can experimental studies pertain to clinical practice?

If better understanding of molecular signalling is to be of value then its effect on tumour cell behaviour must be discerned. This is an exacting task. Human biology is immensely complex and our understanding of tumour biology remains rudimentary. This makes it difficult to ascertain the function of single molecules and has necessitated a reductionist approach utilising experimental models. The invasion studies exemplify this, focusing on a single cellular function, invasion, and a solitary molecule, EGF receptor. However, invasion is only one element of the composite that is metastasis, and isolation of receptor function is untenable for the very reason that its interaction with other factors defines its function. This means that interpretation of such studies is fraught with difficulty and must be considered in the light of the study's limitations. These limitations also require that we continue clinical studies since these will promulgate appropriate experimental questions. EGF receptor, down regulated in nearly all breast cancers, might appear a poor therapeutic target. However, the invasion studies demonstrate that it may remain a functionally valid target. From the clinical study, its relationship with tumour size fits with the mitogenic signal that is known to result from EGF receptor stimulation. This, together with this receptors confirmed predictive value on survival, suggest that abrogation of EGF receptor signalling could be a useful therapeutic goal. This has been substantiated by experimental work utilising immunotherapy and tyrosine kinase inhibitors. Work that exemplifies the improvements in patient management that could be achieved with an improved understanding of molecular signalling.

REFERENCES

Aaronsson SA 'Growth factors and cancer' Science 254:1146-53, 1991

Akasaka T, van Leeuwen RL, Yoshinga IG, Mihm MC and Byers HR 'Focal adhesion kinase (p125FAK) expression correlates with motility of human melanoma cell lines' J Invest. Dermatol. 105:104-8, 1995

Alberts B, Bray D, Lewis J, Raff M, Roberts K and Watson JD 'Molecular biology of the cell' p1019, 1989

Albini A, Iwamoto Y, Kleinman HK, Martin GR, Aaronson SA, Kozlowski JM and McEwan RN. 'A rapid *in vitro* assay for quantitating the invasive potential of tumour cells' Cancer Res 47: 3239-3245, 1987

Alderson MR, Hamlin I and Staunton MD 'The relative significance of prognostic factors in breast carcinoma' Br J Cancer 25,646-56, 1971

Allard JD, Chang HC, Herbst R, McNeill H and Simon MA 'The SH2-containing tyrosine phosphatase corkscrew is required during signalling by sevenless, Ras1 and Raf' Development 122,1137-46, 1996

Artega CL, Tandon AK, Von Hoff CD and Osborne CK 'Transforming growth factor-β: potential autocrine growth inhibitor of oestrogen receptor negative breast cancer cells' Cancer Res 45: 3898-3904, 1988

Bae SN, Arand G, Azzam H, Pavasant P Torri J, Frandsen T and Thompson EW 'Molecular and cellular analysis of basement membrane invasion by human breast cancer cells in Matrigel based in-vitro assays' Breast Cancer Res and Treatment 24:241-255, 1993.

Balslev I, Axelsson CK, Zedeler K, Rasmussen BB, Cartensen B and Mouridsen B 'The Nottingham prognostic index applied to 9149 patients from the studies of the Danish breast cancer co-operative group (DBCG)' Breast Cancer Res and Treatment 32:281-90, 1994

Bartel DP, Sheng M, Lau LF and Greenberg ME 'Growth factors and membrane depolarisation activate distinct programs of early response gene expression: dissociation of *fos* and *jun* induction' Genes Dev 3:304-13, 1989

Basson CT and Sidebottom E 'An in vitro study of invasion to compare metastatic and non-metastatic cells' Treatment of Metastases: problems and prospects Ed. Hellman K, Eccles SA, pp243-245 Taylor and Francis, Philidelphia 1985

Bastholm L, Nielsen MH, De May J, Dano K, Brunner N, Hoyer-Hansen G, Ronne E and Elling F 'Confocal fluorescence microscopy of the urokinase plasminogen activator receptor and cathepsin D in human MDA-MB-231 breast cancer cells migrating in reconstituted basement membrane' Biotechnic.&.Histochemistry 69: 61-67, 1994

Bates SE, Davidson N, Valverius EM, Freter C, Dickson RB, Tam J, Kudlow J, Lippman ME and Salomon DS 'Expression of transforming growth factor α and its messenger ribonucleic acid in human breast cancer: its regulation by oestrogen and its possible functional significance' Mol Endocrinol 2:543-55, 1988

Bates SE, Valverius EM, Ennis BW, Bronzert DA, Sheridan JP, Stampfer M, Mendelsohn J, Lippman ME and Dickson RB 'Expression of the TGF-alpha/EGF receptor pathway in normal human breast epithelial cells' Endocrinol 126:596-607, 1990

Battaglia F, Scambia G, Rossi S, Panici PB, Bellantrone R, Polizzi G, Querzoli P, Negrini R, Iacobelli S, Crucitti F and Mancuso S 'Epidermal growth factor receptor in human breast cancer: correlation with steriod hormone receptors and axillary lymph node involvement' Eur J Cancer Clin Oncol 24(11):1685-90, 1988

Baugnet-Mahieu L and Lamaire M 'Expression of epidermal growth factor receptor and C-erbB-2 oncoprotein in human tumours' Eur J Cancer 26:181(Abstract 138), 1990

Beatson AT 'On the treatment of inoperable cases of carcinoma of the mamma: suggestions for a new method of treatment with illustrative cases' Lancet 2:104-7, 1896

Benraad TJ and Foekens JA. 'Hydroxyapatite assay to measure epidermal growth factor receptor in human primary breast tumours' Ann Clin Biochem 27: 272-3, 1990

Beviglia L, Matsumoto K, Lin CS, Ziober BL and Kramer RH 'Expression of c-Met/HGF receptor in human breast carcinoma: correlation with tumour progression' Int J Cancer 74(3):301-9, 1997

Bissell DM,Arenson DM, Maher JJ and Roll FJ 'Support of cultured hepatocytes by a laminin rich gel. Evidence for a functionally significant subendothelial matrix in normal rat liver' J. Clin. Invest. 79:801-12, 1987

Bloom HJG and Richardson WW 'Histologic grading and prognosis in breast cancer' Br J Cancer 11:359-77, 1957

Bloom HJG and Firld JR 'Impact of tumour grade and host resistance on survival of women with breast cancer' Cancer 28:4580-9, 1971

Bolufer P, Miralles F, Rodriguez A, Vasques C, Lluch A, Garcia-Conde J and Olmos T 'Epidermal growth factor receptor in human breast cancer: correlation with cytosolic and nuclear ER receptors and with biological and histological tumour characteristics' Eur J Cancer 26:283-90, 1990

Boonstra J, Moolenaar WH, Harrison PH, Moed P, van der Saag PT and de Laat SW 'Ionic responses and growth stimulation by nerve growth factor and epidermal growth factor in rat phaeochromocytoma (PC12) cells' J Cell Biol 97: 92-8, 1983

Bradford M 'A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein dye binding' Anal Biochem 72:248-54, 1976

Brewster D, Crichton J and Muir C 'How accurate are Scottish Cancer registration data' Br J Cancer 70:954-9, 1994

Brockdoff G, Wieland W, Woelfl G Hofstaedter F and Knuechel R 'Evaluation of flow-cytometric three-parameter analysis for EGFR quantification and DNA assessment in human bladder cancers' Virchows Archiv 432(1):77-84, 1998

Brunton V, Ozanne BW, Paraskeva C and Frame MC 'A role for epidermal growth factor receptor, c-Src and focal adhesion kinase in an in vitro model for the progression of colon cancer' Oncogene 14(3):283-93, 1997

Buchdunger E, Trinks U, Mett H, Regenass U, Muller M, Meyer T, McGlynn E, Pinna L, Traxler P and Lydon N. '4,5-Dianilinophthalimide: A protein-tyrosine kinase inhibitor with selectivity for the epidermal growth factor receptor signal transduction pathway and a potent *in vivo* antitumour activity' Proc Natl Acad Sci USA, 91;2334-2338, March 1994

Burtin P, Chavanel G, Foidart JM and Martin E 'Antigens of basement membrane in the peritumoral stroma in human colon adenocarcinomas: an immunofluorescence study' Int J Cancer 30:13-8, 1982

Carpenter G and Cohen S 'Epidermal growth factor' Annu Rev Biochem 48:193-216, 1979

Carpenter G 'Epidermal growth factor is a major growth promoting hormone in milk' Science 210:198-9, 1980

Catoretti G, Andreola S, Clemente C, D'Amato L and Rilke F 'Vimentin and p53 expression in epidermal growth factor receptor positive, oestrogen receptor negative breast carcinomas' Brit J Cancer 57: 353-357, 1988

Charpin C, Devictor B, Bonnier P, Andrac L, Lavaut M, Allasia C and Piana L 'Epidermal growth factor receptor in breast cancer: Correlation of quantitative immunocytochemical assays to prognostic factors' Breast Cancer Res. Treat. *25*: 203-210, 1993.

Chen P, Gupta K and Wells A 'Cell movement elicited by epidermal growth factor receptor requires kinase and autophosphorylation but is separable from mitogenesis' J Cell Biol 124:547-555, 1994

Chen P, Xie H, Sekar MC, Gupta KB and Wells A 'Epidermal growth factor receptor-mediated cell motility: phospholipase C activity is required, but MAP kinase activity is not sufficient for induced cell movement' J Cell Biol 127:847-57, 1994[1]

Chen WS, Lazar CS, Poenie M, Tsien RY, Gill GN and Rosenfeld MG 'Requirement for the intrinsic protein tyrosine kinase in the immediate and late actions of the EGF receptor' Nature 328:820-3, 1987

Chiquet-Ehrismann R, Kalla P and Pearson CA 'Participation of tenascin and transforming growth factor-β in reciprocal epithelial-mesenchymal interactions of MCF-7 cells and fibroblasts' Cancer Res 49: 4322-4325, 1989

Chrysogelos SA and Dickson RB 'EGF receptor expression, regulation, and function in breast cancer' Breast Cancer Res. Treat. 29 :29-40, 1994.

Ciardiello F, Kim N, Liscia DS, Bianco C, Lidereau R, Merlo G, Callahan R, Greiner J, Szpak C, Kidwell W, Schlom J and Salomon DS 'Transforming growth factor α (TGFα) mRNA expression in human breast carcinomas and TGFα activity in the effusions of breast cancer patients' J Natl Cancer Inst 81:1165-71, 1989

Ciardiello F, Valverius EM, Colucci-D'Amato GL, Kim N, Bassin RH and Salomon DS 'Differential growth factor expression in transformed mouse NIH-3T3 cells' J Cell Biochem 42:45-57, 1990

Ciardiello F, McGready ML, Kim N, Basolo F, Hynes NE, Langton B, Yokozaki H, Saeki T, Elliot J, Masui H, Mendelsohn J, Soule H, Russo J and Salomon DS 'Transforming growth factor-α expression is enhanced in human mammary epithelial cells transformed by an activated c-Ha-*ras* proto-oncogene but not by the c-*neu* proto-oncogene, and overexpressing of the transforming growth factor-α complementary DNA leads to transformation' Cell Growth Diff 1:407-20, 1990

Collins VP 'Amplified genes in human gliomas' Sem Cancer Biology 4:27-32, 1993

Cook PW, Pittelkow MR, Keeble WW, Graves-Deal R, Coffey RJ Jr and Shipley GD 'Amphiregulin messenger RNA is elevated in psoriatic epidermis and gastrointestinal carcinomas' Cancer Res 52:3224-7, 1992

Cooke T 'The clinical application of oestrogen receptor analysis in early carcinoma of the breast' Ann Roy Coll Surg Engl 64:165-70, 1982

Coombes RC, Murray P and Luqmani Y 'The prognostic significance of transcripts of transforming growth factor alpha and beta and epidermal growth factor receptor in primary breast cancer' Eur J Cancer 26:159(Abstract 49), 1990

Connell ND and Rheinwald JG 'Regulation of the cytoskeleton in mesothelial cells: reversible loss of keratin and increase in vimentin during rapid growth in culture' Cell 34: 245-253, 1983

Costa S, Stamm H, Almendral A, Ludwig H, Wyss R, Fabbro D, Ernst A, Takahashi A and Eppenberger U 'Predictive value of EGF receptor in breast cancer' Lancet 2:1258, 1988

Countaway JL, McQuilken P, Girones N and Davies RJ 'Multisite phosphorylation of the epidermal growth factor receptor' J Biol Chem 265:3407-16, 1990

Cresson DH, Beckman WC, Tidwell RR, Geratz JD and Siegal GP 'In vitro inhibition of human sarcoma cells' invasive ability by bis(5-amidino-2-benz(midazolyl)methane - A novel estero-protease inhibitor' Am J Pathol 123:46-56, 1986

Darcy K, Black JD, Hahm HA and Ip MM 'Mammary organoids from immature virgin rats ubdergo ductal and alveolar morphogenesis when grown within a constituted basement membrane' Exp Cell Res 196:49-65, 1991

Davidson EK 'Carcinoma in the mammary lobule and its origin' Edinburgh Medical Journal XL 57-82, 1933.

Davidson NE, Gelmann EP, Lippman ME and Dickson RB 'Epidermal growth factor receptor gene expression in oestrogen receptor positive and negative human breast cancer cell lines' Mol Endocrinol 1:216-23, 1987

Davies DE and Chamberlain SG 'Targeting the epidermal growth factor receptor for therapy of carcinomas' Biochem Pharmacol 51:1101-10, 1996

De Sombre ET and Jensen EV 'Estrophilin assays in breast cancer: quantitative features and application to the mastectomy specimen' Cancer 46:2783-8, 1980

Di Fiore PP, Pierce JH, Fleming TP, Hazan R, Ullrich A, King CR, Schlessinger J and Aaronson SA 'Overexpression of the human EGF receptor confers an EGF dependent transformed phenotype to NIH 3T3 cells' Cell 51:1063-70, 1987

Di Marco E, Pierce JH, Fleming TP, Kraus MH, Molloy CJ, Aaronson SA and DiFiore PP 'Autocrine interaction between TGF-alpha and the EGF receptor: quantitative requirements for induction of the malignant phenotype' Oncogene 4:831-838, 1989

Dittadi R, Donisi PM, Brazzale A, Cappellozza L, Bruscagnin G and Gion M 'Epidermal growth factor receptor in breast cancer. Comparison with non malignant breast tissue' Brit. J. Cancer 67 :7-9, 1993.

Dobashi Y and Stern DF 'Membrane anchored forms of EGF stimulate focus formation and intercellular communication' Oncogene 6:1151-9, 1991

Domagala W, Lasota J, Dukowicz A et al 'Vimentin expression appears to be associated with poor prognosis in node-negative ductal NOS breast carcinomas' Am J Pathol 137: 1299-1304, 1990

Downward J, Yarden Y, Mayes E, Scrace G, Totty N, Stockwell P, Ullrich A, Schlessinger J and Waterfield MD 'Close similarity of epidermal growth factor receptor and v-erbB oncogene protein sequences' Nature 307:521-7, 1984

Downward J, Parker P and Waterfield MD 'Autophosphorylation sites on the epidermal growth factor receptor' Nature 311:483-5, 1984[1]

Earp HS, Dawson TL, Li X and Lu H 'Heterodimerisation and functional interaction between EGF receptor family members: A new signalling paradigm with implications for breast cancer research' Breast Cancer Res. Treat. 35: 115-132, 1995.

Elston CW and Ellis IO 'Pathological prognostic factors in breast cancer: I The value of histological grade in breast cancer: experience from a large study with long term follow up' Histopathology 19 :403-410, 1991.

Fisher B, Bauer M, Wickerham L, Redmond CK and Fisher ER 'Relation of number of positive axillary nodes to the prognosis of patients with primary breast cancer, an NSAPB update' Cancer 52:1551-7, 1983

Foekens JA, Beex LVAM, Guerts-Moespot A, Heuvel JJTM, Kienhuis CBM and Benraad TJ 'Epidermal growth factor receptor-negative tumours are predominantly confined to the subgroup of oestradiol receptor positive human primary breast cancers' Cancer Res 51:4544-8, 1991

Foekens JA, Portengen H, van Putten WLJ, Trapman AMAC, Reubi J-C, Alexieva-Figusch J and Klijn JGM 'Prognostic value of receptors for insulin-like growth factor 1, somatostatin, and epidermal growth factor in human breast cancer' Cancer Res 49:7002-9, 1989

Fox SB, Smith K, Hollyer J, Greenall M, Hastrich D and Harris AL 'The epidermal growth factor receptor as a prognostic marker: results of 370 patients and review of 3009 patients' Breast Cancer Res Treat 29:41-9, 1994

Fox SB, Leek RD, Smith K, Hollyer J, Greenall M and Harris AL 'Tumour angiogenesis in node negative breast carcinomas-relationship with epidermal growth factor, oestrogen receptor and survival' Breast Cancer Res Treat 29:109-16, 1994[1]

Fidler IJ 'Tumour heterogeneity and the biology of cancer invasion and metastasis' Cancer Res 38: 2651-2660, 1978

Fidler IJ 'Critical factors in the biology of human cancer metastasis: twenty eighth G H A Clowes memorial lecture' Cancer Res 50: 6130-6138, 1990

Fidler IJ and Radinsky R 'Editorial: Genetic control of cancer metastasis' J Natl Cancer Inst 82:166-8, 1990[1]

Finzi E, Fleming T, Segatto O, Pennington CY, Derynck R and Aaronson SA 'The human transforming growth factor type alpha coding sequence is not a directly acting oncogene when overexpressed in NIH/3T3 cells' Proc Natl Acad Sci USA 84:3733-7, 1987

Finzi E, Fleming T and Pierce JH 'Retroviral expression of transforming growth factor alpha does not transform fibroblasts or keratinocytes' J Invest Dermatol 95:382-7,1990

Fitzpatrick SL, Brightwell J, Wittliff JL, Barrows GH and Schultz GS 'Epidermal growth factor binding by breast tumour biopsies and relationship to oestrogen receptor and progesterone receptor levels' Cancer Res 44:3448-53, 1984

Gabor H and Weiss L 'Mechanically induced trauma suffered by cancer cells in passing through pores in polycarbonate membranes' Invasion Metas 5: 71-83, 1985

Gabor H and Weiss L 'Perturbations in cancer cell deformability and resistance to shear forces' Invasion Metas 6(3): 166-179, 1986

Gasparini G, Reitano M, Bevilaqua P, Meli S, Pozza F and Santini G 'Relationship of the epidermal growth factor receptor to the growth fraction (KI-67 antibody) and the flow cytometric S-phase as cell kinetics parameters, in human mammary carcinomas' Anticancer Res. 11: 1597-1604, 1991

Gasparini G, Bevilacqua P, Pozza F, Meli S, Boracchi P, Marubini E and Sainsbury JRC. 'Value of epidermal growth factor status compared with growth fraction and other factors for prognosis in early breast cancer' Brit. J. Cancer 66: 970-976, 1992.

Gill GN, Bertics PJ and Stanton JB 'Epidermal growth factor and its receptor' Mol Cell Endocrinol 51:169-86, 1987

Golembo M, Raz E and Shilo B-Z 'The drosophila embryonic midline is the site of Spitz processing, and induces activation of the EGF receptor in the ventral ectoderm' Development 122,3363-70, 1996

Gorbunoff MJ The interactions of proteins with hydroxyapatite. I Role of protein charge and structure' Anal Biochem 136:425-32, 1984

Gorbunoff MJ and Timasheff SN 'The interactions of proteins with hydroxyapatite. III Mechanism' Anal Biochem 136:440-5, 1984[1]

Greenberg ME, Greene LA and Ziff EB 'Nerve growth factor and epidermal growth factor induce rapid transient changes in proto-oncogene transcription in PC12 cells' J Biol Chem 260:14101-10, 1985

Gregory H 'Isolation and structure of urogastrone and its relationship to epidermal growth factor' Nature 257:325-7, 1975

Gregory H, Thomas CE, Willshire TR, Young JA, Andersson H, Baildam A and Howell A 'Epidermal growth factor and transforming growth factor α in patients with breast tumours' Br J Cancer 59:605-9, 1989

Grimaux M, Romain S, Remvikos Y, Martin PM and Magdelenat H 'Prognostic value of epidermal growth factor receptor in node-positive breast cancer' Breast Cancer Res Treat 14:77-90, 1989

Grimaux M, Mady E, Remvikos Y, Laine-Bidron C and Magdelenat H 'A simplified immuno-enzymatic assay of the epidermal growth factor receptor in breast tumours: evaluation in 282 cases' Int J Cancer 45:255-62, 1990

Guerin M, Gabillot M, Mathieu M-C, Travagli J-P, Spielmann M, Andrieu N and Riou G 'Structure and expression of C-erbB-2 and EGF receptor genes in inflammatory and non-inflammatory breast cancer: prognostic significance' Int J Cancer 43:201-8, 1989

Hainsworth PJ, Henderson MA, Stillwell RG and Bennett RC 'Comparison of EGF-R, C-erbB-2 product and *ras* p21immunohistochemistry as prognostic markers in primary breast cnacer' Eur J Surg Oncol 17:9-15, 1991

Haley J, Whittle N, Bennett P, Kinchington D, Ullrich A and Waterfield M 'The human EGF receptor gene: structure of the 110kb locus and identification of sequences regulating its transcription' Oncogene Res 1:375-96, 1987

Hamburger AW, White CP and Brown RW 'Effect of epidermal growth factor on proliferation of human tumor cells in soft agar' J Natl. Cancer Inst 67:825-30, 1981

Harris AL, Nicholson S, Sainsbury JRC, Farndon J and Wright C 'Epidermal growth factor receptors in breast cancer: association with early relapse and death, poor response to hormones and interaction with neu' J. Steroid Biochem. 34: 123-131, 1989

Harris AL 'What is the biological, prognostic and therapeutic role of the EGF receptor in human breast cancer' Breast Cancer Res Treat 29:1-2, 1994

Hart IR and Fidler IJ 'An in-vitro quantitative assay for tumour cell invasion' Cancer Res 38: 3218-3224, 1978

Haybittle JL, Blamey RW, Elston CW, Johnson J, Doyle PJ, Campbell FC, Nicholson RI and Griffiths K 'A prognostic index in primary breast cancer' Br J Cancer 45,361-6, 1982

Heisermann GJ, Wiley HS, Walsh BJ, Ingraham HA, Fiol CJ and Gill GN 'Mutational removal of the thr669 and ser671 phosphorylation sites alters substrate specificity and ligand induced internalisation of the epidermal growth factor receptor' J Biol Chem 265:12820-7, 1990

Heldin CH, Betsholtz C, Claesson-Welsh L and Westermark B 'Subversion of growth regulatory pathways in malignant transformation' Biochem Biophys Acta 907:219-44, 1987

Helin K, Velu T, Martin P, Vass WC, Allevato G, Lowy DR and Beguinot L 'The biological activity of the human epidermal growth factor receptor is positively regulated by its C-terminus tyrosines' Oncogene 6:825-32, 1991

Hendler FJ and Ozanne BW 'Human squamous cell lung cancers express increased epidermal growth factors' J. Clin Invest 74: 647-651, 1984.

Hendrix MJC, Seftor EA, Seftor REB and Fidler IJ 'A simple quantitative assay for studying the invasive potential of high and low human metastatic variants' Cancer Letters 38: 137-147, 1987

Hendrix MJC, Gehlsen KR, Wagner HN, Rodney SR, Misiorowski RL, Meyshens FC jr 'In vitro quantification of melanoma tumour cell invasion' Clin Exp Metastasis 3:221-33, 1985

Hendrix MJC, Seftor EA, Seftor REB, Misiorowski PZ, Sundershan and Welsh DR 'Comparison of tumour cell invasion assay: human amnion versus reconstituted basement membrane barriers' Invasion Metastasis 9: 278-297, 1989

Hennigan RF, Hawker KL and Ozanne BW 'Fos-transformation activates genes associated with invasion, Oncogene' 9:3591-3600, 1994

Holt JT, Jensen RA and Page DL 'Histopathology: old principles and new methods' Cancer Surv 18:115-33, 1993

Hommel U, Dudgeon TJ, Fallon A, Edwards RM and Campbell IA 'Structure-function relationships in human epidermal growth factor studied by site directed mutagenesis and ^{1}H NMR' Biochemistry 30:8891-8, 1991

Honegger AM, Dull TJ, Felder S, Van Obberghen E, Bellot F, Szapary D, Schmidt A, Ullrich A and Schlessinger J 'Point mutation at the ATP binding site of EGF receptor abolishes protein tyrosine kinase activity and alters cellular routing' Cell 51:199-209, 1987

Honegger AM, Szapary D, Schmidt A, Lyall R, Van Obberghen E, Dull TJ, Ullrich A and Schlessinger J 'A mutant epidermal growth factor receptor with defective protein tyrosine kinase is unable to stimulate proto-oncogene expression and DNA synthesis' Mol Cell Biol 7:4568-71, 1987[1]

Honegger AM, Dull TJ, Szapary D, Komoriya A, Kris R, Ullrich A and Schlessinger J 'Kinetic parameters of the protein tyrosine kinase activity of the EGF receptor mutants with individually altered autophosphorylation sites' EMBO J 7:3053-60, 1988

Howell A, Barnes DM, Harland RN, Redford J, Bramwell VH, Wilkinson MJ, Swindell R, Crowther D and Sellwood RA 'Steriod-hormone receptors and survival after first relapse in breast cancer' Lancet 1(8377):588-91, 1984

Huff K, End D and Guroff G 'Nerve growth factor induced alteration in the response of PC12 phaeochromocytoma cells to epidermal growth factor' J Cell Biol 88:189-98, 1981

Hujanen ES and Terranova VP. 'Migration of tumour cells to organ derived chemoattractants' Cancer Res. 45: 3517-3521, 1985

Ilic D, Furuta Y, Kanazawa S, Takeda N, Sobue K, Nakatsuji N, Nomura S, Fujimoto J, Okada M, Yamamoto T and Aizawa S 'Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice' Nature 377:539-544, 1995

Imamato A, Beltran LM and DiGiovanni J 'Evidence for autocrine / paracrine growth stimulation by TGF-α during the process of skin tumour promotion' Mol Carcinog 4:52-60, 1991

Imamura F, Horai T, Mukai M, Shinkai K and Akedo H 'Serum requirement for in vitro invasion by tumour cells' Jpn J Cancer Res 82: 493-496, 1991

Imamura H, Takao S and Aikou T 'A modified invasion-3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide assay for quantitating tumour cell invasion' Cancer Res. 54: 3620-3624, 1994

Inoue S, Leblond CP and Laurie GW 'Ultrastructure of Reichert's membrane, a multilayered basement membrane in the parietal wall of the rat yolk sac' J Cell Biol. 97:1524-37, 1983

Ioannidis JP, Cappelleri JC and Lau J 'Issues in comparisons between meta-analyses and large trials' JAMA 279 (14):1089-93, 1998

Jackoew SB, Kondaiah P, Flanders KC, Thompson NL, Dillard PJ, Sporn MB and Roberts AB 'Increased expression of growth factor mRNA's accompanies viral transformation of rodent cells' Onc Res 2:135-48, 1988

Johnson GR, Kannan B, Shoyab M and Stromberg K 'Amphiregulin induces tyrosine phosphorylation of the epidermal growth factor receptor and p185$^{erbB\text{-}2}$. Evidence that amphiregulin acts exclusively through the epidermal growth factor receptor at the surface of human epithelial cells' J Biol Chem 268:2924-31, 1993

Jones PA and DeClerck YA 'Destruction of extracellular matrices containing proteoglycans, elastin and collagen by metastatic human tumour cells' Cancer Res. 40: 3222-3227, 1980

Jones JL, Royall JE and Walker RA 'E-Cadherin relates to EGFR expression and lymph node metastasis in primary breast carcinoma' Brit J Cancer 74:1237-41, 1996

Kameyama S, Kawamata H, Pastan I and Oyasu I 'Cytotoxic effect of fusion protein from transforming growth factor α and pseudomonas exotoxin on rat and human bladder carcinoma cells in-vitro' J Cancer Res Clin Oncol 120:507-12, 1994

Karabali-Dalamaga S, Souhami RL, O'Higgins NJ, Soumilas A and Clark CG 'Natural history and prognosis of recurrent breast cancer' Br Medical J 2(6139):730-3, 1978

Karameris A, Kanavaros P, Aninos D, Gorgoulis V, Mikou G, Rokas T, Niotis M and Kalogeropoulos N 'Expression of epidermal growth factor (EGF) and epidermal growth factor receptor (EGFR) in gastric and colorectal carcinomas. An immunohistological study of 63 cases' Pathology, Research & Practice. 189(2):133-7, 1993 Mar.

Kenny N, Johnson GR, Selvam MP, Kim N, Qi C, Saeki T, Brandt R, Ciardiello F, Shoyab M, Plowman GD, Day A, Normanno N and Salomon DS 'Transforming growth factor alpha and amphiregulin as autocrine growth factors in nontransformed immortalised 184A1N4 human mammary epithelial cells' Mol Cell Diff 1:163-84, 1993

Khazaie K, Dull TJ, Graf T, Schlessinger J, Ullrich A, Beug H and Venstrom B 'Truncation of the human EGF receptor leads to differential transforming potentials in primary avian fibroblasts and erythroblasts' EMBO J 7:3061-71, 1988

Khaziae K, Schirrmacher V and Lichtner RB 'EGF receptor in neoplasia and metastasis' Cancer Metastasis Rev 12:255-274, 1993

Kinsel LB, Szabo E, Greene GL, Konrath J, Leight GS and McCarty KS jr. 'Immunohistochemical assessment of oestrogen receptors as a predictor of prognosis in breast cancer patients: comparison with quantitative biochemical methods' Cancer Res 49:1052-6, 1989

Kirk J, Carmichael J, Stratford IJ and Harris AL 'Selective toxicity of TGF-α-PE40 to EGFR positive cell lines: selective protection of loe EGFR expressing cell lines by EGF' Brit J Cancer 69:988-994, 1994

Kleinhaus CBM, Heuvel JJTM, Ross HA, Swinkels LMJW, Foekens JA and Benraad ThJ 'Six methods for direct radioiodination of mouse epidermal growth factor compared: Effect of non-equivalence in binding behaviour between labeled and unlabeled ligand' Clin Chem 37:1749-1755, 1991

Kleinmann HK, Klebe RJ and Martin GR 'Role of collagenous matrices in the adhesion and growth of cells' J Cell Biol 88:473-82, 1981

Kleinmann HK, McGarvey ML, Liotta LA, Robey PG, Tryggvason K and Martin GR 'Isolation and characterisation of type IV procollagen, laminin amd heparan sulphate proteoglycan from the EHS sarcoma' Biochemistry 21: 6188-6193, 1982

Kleinmann HK, McGarvey ML, Hassell JR, Star VL, Cannon FB, Laurie GW and Martin GR 'Basement membrane complexes with biological activity' Biochemistry 25: 312-318, 1986

Klijn JGM, Berns PMJJ, Schmitz PIM and Foekens JA 'The clinical significance of epidermal growth factor receptor (EGF-R) in human breast cancer: A review on 5232 patients' Endocrine Reviews 13: 3-17, 1992

Koch JH, Fifis T, Bender VJ and Moss BA 'Molecular species of epidermal growth factor carrying immunosuppressive activity' J Cell Biochem 25:45-59, 1984

Koenders PG, Beex LVAM, Geurts-Moespot A, Heuvel JJTM, Kleinhuis CBM and Benraad TJ 'Epidermal growth factor receptor-negative tunours are predominantly confined to the sub-group of Estradiol receptor-positive human primary breast cancers' Cancer Res 51:4544-8, 1991.

Koenders PG, Faverly D, Beex LVAM, Bruggink EDM, Kienhuis CBM and Benraad ThJ 'Epidermal growth factor receptors in human breast cancer: a plea for standardisation of assay methodology' Eur. J. Cancer 28: 693-697, 1992.

Kramer RH and Vogel KG 'Selective degradation of basement membrane macromolecules by human breast cancer cell lines' J Natl Cancer Inst 72: 889-899, 1984

Kramer RH, Bensch KK and Wong J 'Invasion of reconstituted basement membrane matrix by metastatic tumour cells' Cancer Res. 46:1980-1989, 1986

Kraus MH, Popescu NC, Amsbaugh C and King CR 'Overexpression of the EGF receptor-related proto-oncogene C-erbB-2 in human mammary tumour cell lines by different molecular mechanisms' EMBO J 6: 605-610, 1987

Laurie GW, Leblond CP and Martin GR. 'Locallisation of type IV collagen, laminin, heparan sulphate proteoglycan and fibronectin to the basal lamina of basement membranes' J Cell Biol 95:340-344, 1982

Leake RE, Foekens JA and Benraad TJ. 'Detailed methodologies of the development of assays for growth factors and growth factor receptors' Commisission of the European Communities, EUR14799EN, 1993

Levitski A and Gazit A 'Tyrosine kinase inhibition: an approach to drug development' Science 267:1782-7, 1995

Lewis S, Locker A, Todd JH, Bell JA, Nicholson R, Elston CW, Blamey RW and Ellis IO 'Expression of epidermal growth factor receptor in breast carcinoma' J Clin Pathol 43:385-9, 1990

Li S, Plowman GD, Buckley SD and Shipley GD 'Heparin inhibition of autonomous growth implicates amphiregulin as an autocrine growth factor for normal human mammary epithelial cells' J Cell Physiol 153:103-11, 1992

Lichtman JW 'Confocal Microscopy', Scientific American, 30-35, August 1994

Lichtner RB, Weidemuth M, Noeske-Jungblut C and Schirrmacher V 'Rapid effects of EGF on cytoskeletal structures and adhesive properties of highly metastatic rat mammary adenocarcinoma cells' Clin Exp Metastasis 11:113-25, 1993

Lin CR, Chen WS, Kruiger W, Stolarsky LS, Weber W, Evans RM, Verma IM, Gill GN and Rosenfeld MG 'Expression cloning of human EGF receptor complementary DNA: gene amplification and three related messanger RNA products in A431 cells' Science 224:843-8, 1984

Lin CR, Chen WS, Lasar CS, Carpenter CD, Gill GN, Evans RM and Rosfeld MG 'Protein kinase C phosphorylation at Thr654 of the unoccupied EGF receptor and EGF binding regulate functional receptor loss by independent mechanisms' Cell 44:839-48, 1986

Liotta LA, Lee WC and Morakis DJ 'New method for preparing large surface of intact basement membrane for tumour invasion studies' Cancer Lett 11: 141-147, 1980

Liotta LA 'Tumour invasion and metastases: role of the basement membrane' Am J Pathol 117: 339-348, 1984

Liotta LA, Rao CN and Wewer UM, 'Biochemical interactions of tumour cells with the basement membrane' Ann Rev Biochem 55: 1037-1057, 1986

Liotta LA 'Tumour invasion and metastases: role of the basement membrane' Cancer Res 46:1-7, 1986[1]

Liotta LA, Steeg PS and Stetler-Stevenson WG 'An imbalance of positive and negative regulation' Cell 64: 327-336, 1991

Lippman ME, Dickson RB, Bates S, Knabbe C, Huff K, Swain S, McManaway M, Bronzert D, Kasid A and Gelman EP 'Autocrine and paracrine growth regulation of human breast cancer' Breast Cancer Res Treat 7:59-70, 1986

Long B, McKibben BM, Lynch M and van den Berg HW 'Changes in epidermal growth factor receptor expression and response to ligand associated with acquired tamoxifen resistance or oestrogen independence in the ZR-75-1 human breast cancer cell line' Br J Cancer 65:865-9, 1992

Lundy J, Scuss A, Stanick D, McCormack ES, Kramer S and Sorvillo JM 'Expression of *neu* protein, EGF and TGFα in breast cancer' Am J Pathol 138, 1527-34, 1991

Macias A, Azavedo E, Perez R, Rutquist LE and Skoog L 'Receptors for epidermal growth factor in human mammary carcinomas and their metastases' Anticancer Res 6:849-51, 1986

Macias A, Azavedo E, Hagerstrom T, Klintenberg C, Perez R and Skoog L 'Prognostic significance of the receptor for epidermal growth factor in human mammary carcinomas' Anticancer Res 7:459-64, 1987

Macias A, Perez R, Hagerstrom T and Skoog L 'Transforming growth factor α in human mammary carcinomas and their metastases' Anticancer Res 10:1115-26, 1990

Mackinnon WB, Hancock R, Dyne M, Russell P and Mountford CE 'Evaluation of an in vitro invasion assay for use on solid tissue samples and cultured cells' Invasion Metastasis 12: 241-252, 1992

Maher P 'Nerve growth factor induces protein-tyrosine phosphorylation' Proc Natl Acad Sci USA 85:6788-91, 1988

Mareel M, Kint J and Meyvisch C 'Methods of study of the invasion of malignant C3H-mouse fibroblasts into embryonic chick heart in vitro' Vichows Archiv B, Cell Pathol 30: 95-111, 1979

Marquardt H, Hunkapiller MW, Hood LE, Twardzik DR, DeLarco JE, Stephenson JR and Todaro GJ 'Transforming growth factors produced by retrovirus transformed rodent fibroblasts and human melanoma cells: amino acid sequence homology with epidermal growth factor' Proc Natl Acad Sci USA 80:4684-8, 1983

Marquardt H, Hunkapiller MW, Hood LE and Todaro GJ 'Rat transforming growth factor type I: structure and relation to epidermal growth factor' Science 223:1079-82, 1984

Massague J 'Epidermal growth factor-like transforming growth factor 2. Interaction with epidermal growth factor receptors on human placenta membrane and A431 cells' J Biol Chem 258:13614-20, 1983

Massague J 'Transforming growth factor-α, A model for membrane anchored growth factors' J Biol Chem 265:21393-6, 1990

Matrisian LM and Hogan BLM 'Growth factor-regulated proteases and extracellular matrix remodeling during mammalian development' Current topics in Developmental biology 24:219-54, 1990

Matsumoto K, Matsumoto K, Nakamura T and Kramer RH 'Hepatocyte growth factor/scatter factor induces tyrosine phosphorylation of focal adhesion kinase (p125FAK) and promotes migration and invasion by oral squamous cell carcinoma cells' J.Biol. Chem. 269:31807-13, 1994

McArdle CS 'Surgical oncology: current concepts and practice' Ed. McArdle CS, pp132-137, Butterworths, London 1990

McGeady ML, Kerby S, Shankar V, Ciardiello F, Salomon D and Seidman M 'Infection with a TGF-α retroviral vector transforms mouse mammary epithelial cells but not normal rat fibroblasts' Oncogene 4:1375-82, 1989

Merlino GT 'Epidermal growth factor receptor regulation and function' Semin Cancer Biol 1:277-84, 1990

Mignatti P, Robbins E and Rifkin DB 'Tumour invasion through the human amniotic membrane: requirement for the proteinase cascade' Cell 47: 487-498, 1986

Miller WR, Ellis IO, Sainsbury JRC and Dixon JM 'ABC of breast diseases, Prognostic factors' Brit Med J 309:1573-1576, 1994

Minckwitz G, Kaufmann H, Schmid H, Goerttler K and Bastert G 'Epidermal growth factor receptor and S-phase fraction as prognosticator combination in node negative primary breast cancer' Breast 2: 229-233, 1993.

Miyachi Y, Terazono T, Nagao N, Shoji T and Irie M 'Epidermal growth factor (EGF) receptors in human chorionic gonadotrophin-producing tumour: transplantation in nude mice and the effect of EGF on tumour growth' J Clin Endocrinol Metab 71:329-34, 1990

Modjtahedi H, Styles J, Box G, Eccles S, Gusterton B and Dean C, 'Antitumour activity of rat monoclonal antibodies to the human receptor for EGF' Mutant Oncogenes: Targets for therapy? Eponetos AA, Lemoine NR (Editors), Chapman Hall Medical (1992)

Modjtahedi H, Eccles S, Box G, Styles J and Dean C 'Immunotherapy of human tumour xenografts overexpressing the EGF receptor with rat antibodies that block growth factor-receptor interaction' Br J Cancer 67:254-261, 1993

Modjtahedi H and Dean C 'The receptor for EGF and its ligands: Expression, prognostic value and target for therapy in cancer' Int. J Oncol 4:277-296, 1994

Moller P, Mechtersheimer G, Kaufmann M, Moldenhauer GMomburg F and Mattfeldt T 'Expression of epidermal growth factor receptor in benign and malignant primary tumours of the breast' Virchows Arch [Pathol Anat] 414:157-64, 1989

Monti E and Sinha BK 'Antiproliferative effect of Genistein and adriamycin against oestrogen dependent and -independent human breast carcinoma cell lines' Anticancer Research 14(3A):1221-6, 1994

Mossmann T 'Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays' J Immunol Methods 65: 55-63, 1983

Munson PJ 'LIGAND: a computerised analysis of ligand binding data' Method Enzymol 92:543-76, 1983

Murayama Y 'Growth-inhibitory effects of epidermal growth factor on human breast cancer and carcinoma of the oesophagus transplanted into nude mice' Ann Surg 211:263-8, 1990

Murphy LC and Dotzlaw H 'Regulation of transforming growth factor α and transforming growth factor β messenger ribonucleic acid abundance in T-47D human breast cancer cells' Mol Endocrinol 3:611-6, 1989

Neal DE, Marsh C, Bennett MK, Abel PD, Hall RR, Sainsbury JR and Harris AL 'Epidermal growth factor receptors in human bladder cancer: Comparison of invasive and superficial tumours' Lancet, 1(8425):366-8, 1985

Nguyen PL, Swnason PE, Jaszcz W, Aeppli DM, Zhang G Singleton TP, Ward S, Dykoski D, Harvey J and Niehans GA 'Expression of epidermal growth factor receptor in invasive transitional cell carcinoma of the urinary bladder: a multivariate survival analysis' American journal of clinical pathology 101:166-76, 1994

Nicholson RI, McClelland RA, Finlay P, Eaton CL, Gullick WJ, Dixon AR, Robertson JFR, Ellis IO and Blamey RW 'Relationship between EGF-R, c-erbB-2 protein expression and Ki67 immunostaining in breast cancer and hormone sensitivity' Eur. J. of Cancer Part A: General Topics 29:1018-1023, 1993.

Nicholson RI, McClelland RA, Gee JMW, Manning DL, Gullick WJ, Cannon P, Robertson JFR, Ellis IO and Blamey RW 'Epidermal growth factor receptor expression in breast cancer: association with response to endocrine therapy' Breast Cancer Res Treat 29:117-125, 1994

Nicholson S, Wright C, Sainsbury JRC, Halcrow P, Kelly P, Angus B, Farndon JR and Harris AL 'Epidermal growth factor receptor as a marker for poor prognosis in node-negative breast cancer patients: neu and tamoxifen failure' J Steriod Biochem Mol Biol 37:811-4, 1990

Nicholson S, Sainsbury JRC, Halcrow P, Kelly P, Angus B, Wright C, Henry J, Farndon JR and Harris AL 'Epidermal growth factor receptor (EGFr): results of a 6 year follow-up study in operable breast cancer with emphasis on the node-negative subgroup' Br J Cancer 63:146-50, 1991

Nickell KA, Halper J and Moses HL 'Transforming growth factor in solid malignant neoplasms' Cancer Res 43:1966-71, 1983

Nigg EA 'Mechanisms of signal transduction to the nucleus' Adv Cancer Res 55:271-310, 1990

Noel A, Calle A, Emonard H, Nugens B, Simar L, Foidart J, Lapiere C and Foidart JM 'Invasion of reconstituted basement membrane matrix is not correlated to the malignant metastatic cell phenotype' Cancer Res 51: 405-414, 1991

Okuda T, Onda M, Tokunaga A and Sugisaki Y 'Stimulatory effect of EGF and inhibitory effect of sialoadenectomy on growth of an EGF receptor-hyperproducing human gastric cancer xenograft in nude mice' Surg Today 24:725-33, 1994

Osborne CK, Hamilton B, Titus G and Livingston RB 'Epidermal growth factor stimulation of human breast cancer cells in culture' Cancer Res 40: 2361-2366, 1980

Osborne CK 'Steroid hormone receptors in breast cancer management' Breast cancer research and treatment 51:227-38, 1998

Ossowski L and Reich E 'Experimental model for quantitative study of metastasis' Cancer Res 40: 2300-2309, 1980

Owens LV, LiHui X, Craven RJ, Dent GA, Weiner TM, Kornberg L, Liu ET and Chance WG 'Overexpression of focal adhesion kinase (p125FAK) in invasive human tumours' Cancer Res. 55:2752-5, 1995

Ozawa S, Ueda M, Ando N, Abe O, Shimizu N 'Epidermal growth factor receptors in cancer tissues of oesophagus, lung, pancreas, colorectum, breast and stomach' Jpn. J. Cancer Res. 79 :1201-1207, 1988.

Paget S 'The distribution of secondary growths in cancer of the breast' Lancet 1:571-573, 1889

Pai LH, Gallo MC, Fitzgerald DJ and Pastan I 'Antitumour activity of transforming growth factor α- Pseudomonas exotoxin fusion protein (TGF-α-PE40)' Cancer Res 51:2808-12, 1991

Paria BC, Das SK, Huet-Hudson YM and Dey SK 'Distribution of transforming growth factor-α precursors in the mouse uterus during the periimplantation period and after steriod hormone treatments' Biol Reprod Dev 50:481-91, 1994

Parish CR, Jakobsen KB and Coombe DR 'A basement membrane permeability assay which correlates with the metastatic potential of tumour cells' Int J Cancer 52: 378-383, 1992

Parkam DH and Jankowski J 'Transforming growth factor α in epithelial proliferative diseases of the breast' J Clin Pathol 45:513-6, 1992

Pathology for Surgeons pp291-318, Ed. Watt PCH, Spence RAJ, Published by Wright 1986

Penar PL, Khoshyomn S, Bhushan A and Tritton TR 'Inhibition of epidermal growth factor receptor-associated tyrosine kinase blocks glioblastoma invasion of the brain' Neurosurgery 40(1):141-51, 1997

Perkins LA, Johnson MR, Melnick MB and Perrimon N 'The nonreceptor protein tyrosine phosphatase corkscrew functions in multiple receptor tyrosine kinase pathways in Drosophila' Dev Biol 180:63-81, 1996

Perrimon N and Perkins LA 'There must be fifty ways to rule the signal: the case of the drosophila EGF receptor' Cell 89:13-16, 1997

Perroteau I, Salomon DS, Debortoli M, Kidwell W, Hazarika P, Pardue R, Dedman J and Tam J 'Immunological detection and quantitation of alpha transforming growth factors in human breast carcinoma cells' Breast Cancer Res Treat 7:201-10, 1986

Plowman GD, Green JM, McDonald VL, Neubauer MG, Disteche CM, Todaro GJ and Shoyab M 'The amphiregulin gene encodes a novel epidermal growth factor-related protein with tumour-inhibitory activity' Mol Cell Biol 10:1969-81, 1990

Poste G, Doll J, Hart IR and Fidler IJ 'In vitro selection of murine B16 melanoma variants with enhanced tissue invasive properties' Cancer Res 40: 1636-1644, 1980

Prigent SA and Lemoine NR 'The type I (EGFR-related) family of growth factor receptors and their ligands' Progress in growth factor research 4:1-24, 1992

Radinsky R, Risin S, Fan D, Dong Z, Bielenberg D, Bucana CD and Fidler IJ 'Level and function of the epidermal growth factor receptor predict the metastatic potential of human colon carcinoma cells' Clin. Cancer Res 1:19-3, 1995

Rajkumar T and Gullick WJ. The type I growth factors in human breast cancer' Breast Cancer Res. Treat. 29(1): 3-9, 1994

Reich R, Thompson EW, Iwamoto Y, Martin GR, Deason JR, Fuller GC and Miskin R. 'Effect of inhibitors of plasminogen activator, serine proteinases and collagenase IV on the invasion of basement membranes by metastatic cells' Cancer Res. 48: 3307-3312, 1988

Reeves JR, Cooke TG, Fenton-Lee D, McNicol AM, Ozanne BW, Richards RC and Walsh A 'Locallisation of EGF receptors in frozen tissue sections by antibody and biotinylated EGF-based techniques' J Histochem Cytochem 42: 307-14, 1994

Reeves JR, Going JJ, Smith G, Cooke TG, Ozanne BW and Stanton PD 'Quantitative radio-immunohistochemical measurements of c-*erb*B-2 receptors in frozen tissue sections' J Histochem Cytochem 44: 1251-9, 1996

Repesh LA 'A new in-vitro assay for quantitating tumour cell invasion' Invasion Matastasis 9: 192-208, 1989

Reubi JC and Torhorst J 'The relationship between somatostatin, epidermal growth factor and steriod hormone receptors in breast cancer' Cancer 64:1254-60, 1989

Rice G and Bevilacqua M 'An inducible endothelial cell surface glycoprotein mediates melanoma adhesion' Science 246:1303-6, 1988

Rios MA, Marcias A, Perez R, Lage A and Skoog L 'Receptors for epidermal growth factor and oestrogen as predictors of relapse in patients with mammary carcinoma' Anticancer Res 8:173-6, 1988

Rios MA, Fernadez A, Tormo B, Quintero S, Perez I, Skoog L and Perez R 'Heterogeneous expression of the EGF receptor in human breast carcinoma' Anticancer Res 12:205-8, 1992

Robertson KW, Reeves JR, Smith G, Keith WN, Ozanne BW, Cooke TG and Stanton PD 'Quantitative estimation of epidermal growth factor receptor and c-erbB-2 in human breast cancer' Cancer Res 56:3823-3830, 1996

Ronnov-Jensen L, Peterson OW and Bissell MJ 'Cellular changes involved in conversion of normal to malignant breast: The importance of the stromal reaction' Annu Rev Physiol, 1995

Roussel MF, Dull TJ, Rettenmier C, Ralph P, Ullrich A and Sherr CJ 'Transforming potential of the c-*fms* proto-oncogene (CSF-1 receptor)' Nature 325:549-52, 1987

Sainsbury JRC, Farindon JR, Sherbet GV and Harris AL 'Epidermal growth factor receptors and oestrogen receptors in human breast cancer' Lancet 1(8425): 364-366, 1985.

Sainsbury JRC, Malcolm AJ, Appleton DR, Farindon JR, and Harris AL 'Presence of epidermal growth factor receptor as an indicator of poor prognosis in patients with breast cancer' J Clin Pathol 38:1225-8, 1985[1]

Sainsbury JRC, Farindon JR, Needham GK, Malcolm AJ and Harris AL 'Epidermal growth factor receptor status as predictor of early recurrence of and death from breast cancer' Lancet 1:1398-1402, 1987

Sainsbury JRC, Nicholson S, Angus B, Farndon JR, Malcolm AJ and Harris AL 'Epidermal growth factor receptor status of histological subtypes of breast cancer' Br J Cancer 58:458-60, 1988

Salomon DS, Kim N, Saeki T and Ciardiello F 'Transforming growth factor-α: an oncodevelopmental growth factor' Cancer Cells 2:389-97, 1990

Sandegren EP, Luetteke NC, Palmiter RD, Brinster RL and Lee DC 'Overexpression of TGF-α in transgenic mice: induction of epithelial hyperplasia, pancreatic metaplasia and carcinoma of the breast' Cell 61:1121-35, 1990

Santon JB, Cronin MT, MacLeod CL, Mendelshon J, Masui H and Gill GN 'Effects of epidermal growth factor receptor concentration on tumorigenicity of A431 cells in nude mice' Cancer Res 46:4701-5, 1986

Savage CR, Hash JH and Cohen S 'Epidermal growth factor' J Biol Chem 248:7669-72, 1973

Savage CR, Inagami T and Cohen S 'The primary structure of epidermal growth factor' J Biol Chem 247:7612-21, 1972

Schlechte W, Brattain M and Boyd D. 'Invasion of extracellular matrix by cultured colon cancer cells: dependence on urokinase receptor display' Cancer Commun 2:173-179, 1990

Schlegel J, Merdes A, Stumm G, Albert FK, Forsting M, Hynes N and Kiessling M 'Amplification of the epidermal growth factor receptor gene correlates with different growth behaviour in human glioblastoma' Int J Cancer 56:72-77, 1994

Schlessinger J 'The epidermal growth factor receptor as a multifunctional allosteric protein' Biochem 27:3119-23, 1988

Schrijvers CT, Mackenbach JP, Lutz JM, Quinn MJ and Coleman MP 'Deprivation and survival from breast cnacer' Br J Cancer 72(3):738-43, 1995

Seeley PJ, Rukenstein A, Connelly JL and Greene LA 'Differential inhibition of nerve growth factor and epidermal growth factor effects on the PC12 phaeochromocytoma line' J Cell Biol 98:417-26, 1984

Shankar V, Ciardiello F, Kim N, Derynck R, Liscia DS, Merlo G, Langton BC, Sheer D, Callahan R, Bassin RH, Lippman ME, Hynes N and Salomon DS 'Transformation of an established mouse mammary epithelial cell line following transfection with a human transforming growth factor alpha cDNA' Mol Carcinogenesis 2:1-11, 1989

Sharma AK, Horgan K, McClelland RA, Douglas-Jones AG, Van Agthoven T, Dorssers LCJ and Nicholson RI 'Duel immunohistochemical assay for oestrogen and epidermal growth factor receptors in tumour cell lines' Histochemical Journal 26(4):306-10, 1994

Shawver LK, Mann E, Ellinger SS, Dugger TC and Arteaga CL 'Ligand-like effects induced by anti-c-erbB-2 antibodies do not correlate with and are not required for growth inhibition of human carcinoma cells' Cancer Research 54(5): 1367-73, 1994 Mar 1

Shiozaki H, Kadowaki T, Doki Y, Inoue M, Tamura S, Oka H, Iwazawa T, Matsui S, Shimaya K Takeichi M and Mori T 'Effect of epidermal growth factor on cadherin-mediated adhesion in a human oesophageal cancer cell line' Brit J Cancer 71(2):250-8, 1995

Shoyab M, McDonald VL, Garrett Bradley J and Todaro GJ 'Ampiregukin: a bi-functional growth-modulating glycoprotein produced by the phorbol 12-myristate 13-acetate treated human breast adenocarcinoma cell line MCF-7' Proc Natl Acad Sci USA 85:6528-32, 1988

Shoyab M, Plowman GD, McDonald VL, Bradley JG and Todaro GJ 'Structure and function of human amphiregulin: A member of the epidermal growth factor family' Science 243:1074-6, 1989

Siegal GP, Barsky SH, Terranova VP and Liotta LA 'Stages of neoplastic transformation of human breast tissue as monitored by dissolution of basement membrane components' Invasion Metast 1:54-65, 1981

Simon N, Noël A and Foidart J 'Evaluation of in vitro reconstituted basement membrane assay to assess the invasiveness of tumout cells' Invasion Metastasis 12: 156-167, 1992

Singletary SE, Baker FL, Spitzer G, Tucker SL, Tomasovic B, Brock WA, Ajani AJ and Kelly AM 'Biological effect of epidermal growth factor on the in-vitro growth of human tumors' Cancer Res 47:403-6, 1987

Skoog L, Marcias A, Azavedo E, Lombardero J, Klintenberg C 'Receptors for EGF and oestradiol and thymidinekinase activity in different histological subgroups of human mammary carcinomas' Br J Cancer 54:271-6, 1986

Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A and McGuire WL 'Human breast cancer: correlation of relapse and survival with amplification of the HER2/*neu* proto-oncogene in human breast and ovarian cancer' Science 235:177-182, 1987

Smart CR, Myers M and Gloechler LA 'Implications from SEER data on breast cancer management' Cancer 41:787-9, 1978

Solic N and Davies DE 'Differential effects of epidermal growth factor and amphiregulin on growth and morphology of two colon carcinoma cell lines' Eur J Clin Invest 25 (suppl. 2):pA53, 1995

Sommers CL, Walker-Jones D Heckford SE, Worland P, Valverius E, Clarke R, McCormack F, Stampfer M, Abularach S and Gelmann EP 'Vimentin rather than keratin expression in some hormone-independent breast cancer cell lines and in oncogene transformed mammary epithelial cells' Cancer Res 49: 4258-4263, 1989

Spitzer E, Grosse R, Kunde D and Schmidt HE 'Growth of mammary epithelial cells in breast-cancer biopsies correlates with EGF binding' Int J Cancer 39:279-82, 1987

Spitzer E, Koepke K, Kunde D and Grosse R 'EGF binding is quantitatively related to growth in node-positive breast cancer' Breast Cancer Res Treat 12:45-9, 1988

Spyratos F, Delarue J-C, Andrieu C, Lidereau R, Champeme M-H, Hacene K and Brunet M 'Epidermal growth factor receptors and prognosis in primary breast cancer' Breast Cancer Res Treat 17:83-9, 1990

Stanton PD, Richards S, Reeves J, Nikolic M, Edington K, Clark L, Robertson G, Souter D, Mitchell R, Hendler FJ, Cooke T, Parkinson EK and Ozanne BW 'Epidermal growth factor receptor expression by human squamous cell carcinomas of the head and neck, cell lines and xenographs' Brit. J Cancer 70:427-433, 1994.

Starkey JR, Hosick HL, Stanford DR and liggitt HD 'Interaction of metastatic tumour cells with bovine lens capsule basement membrane' Cancer Res 44: 1585-94, 1984

Steele RJ, Forrest AP, Gibson T, Stewart HJ, Chetty U. 'The efficacy of lower axillary sampling in obtaining lymph node status in breast cancer: a controlled randomized trial' Br J Surgery. 72(5):368-9, 1985

Stewart JF, King RJB, Sexton SA, Mills RR, Rubens RD and Hayward JL 'Oestrogen receptors, sites of metastatic disease and survival in recurrent breast cancer' Eur J Cancer 17:449-53, 1981

Stoscheck CM and King LE jr 'Role of epidermal growth factor in carcinogenesis' Cancer Res 46:1030-7, 1986

Streuli CH, Bailey N and Bissell MJ 'Control of mammary epithelial differentiation; basement membrane induces tissue specific gene expression in the abscence of cell-cell interaction and morphological parity' J Cell Biol 115:1383-95, 1991

Stroppelli MP, Tachetti C, Cubellis MV, Corti A, Hearing VJ, Cassani G, Appella A and Blasi F 'Autocrine saturation of pro-urokinase receptors on human A431 cells' Cell 45: 675-684, 1986

Sullinen P, Haapasalo H, Kerttula T, Rantala I, Kalimo H, Collan Y, Isola J and Helin H 'Sources of variation in the assessment of cell proliferation using proliferating cell nuclear antigen immunohistochemistry' Analytical and Quantitative Cytology and Histology 16(4): 261-8, 1994

Szollosi J, Balazs M, Feuerstein B, Benz C and Waldman F '*Erb*B-2 (HER2/*neu*) gene copy number, p185^{HER-2} overexpression, and intratumour heterogeneity in human breast cancer' Cancer Res 55: 5400-7, 1995

Takeichi M 'The Cadherins: cell-cell adhesion molecules controlling animal morphogenesis' Development 102:639-55, 1988

Taniguchi S, Tatsuka M, Nakamatsu K, Inoue M, Sadono H, Okazaki H, Iwamoto H and Baba T 'High invasiveness associated with augmentation of motility in a *fos* transferred highly metastatic rat 3Y1 cell line' Cancer Research, 49:6738-41, 1989

Taylor-Papadimitriou J, Berdichevsky F, D'Souza B and Burchell J 'Human models of breast cancer' Cancer Surveys 16: 59-78, 1993

Terranova VP, Hujanen ES and Martin GR.'Basement membrane and the invasive activity of metastatic tumour cells' J Natl Cancer Inst. 77: 311-316, 1986

Terranova VP, Hujanen ES, Loeb DM, Martin GR, Thornburg L and Gluushko V. 'Use of a reconstituted basement membrane to measure cell invasiveness and select for highly invasive tumour cells' Proc Natl Acad Sci USA 83: 465-469, 1986[1]

Thompson EW, Paik S, Brünner N, Sommers CL, Zugmaier G, Shima TB, Torri J, Donahue S, Lippman ME, Martin GR and Dickson RB 'Association of increased basement membrane invasiveness with absence of estrogen receptor and expression of vimentin in human breast cancer cell lines' J Cell Physiol 150: 534-544, 1992

Thorne HJ, Jose DG, Zhang H, Dempsey PJ and Whitehead RH 'Epidermal growth factor stimulates the synthesis of cell-attachment proteins in human breast cancer cell line PMC42' Int J Cancer 40:207-212, 1987

Timpl R, Rhode H, Robey PG, Rennard SI, Foidart JM and Martin GR. 'Laminin - a glycoprotein from basement membrane' J. Biol. Chem. 254: 9933-9937, 1979

Tischler AS and Greene LA Nerve growth factor-induced process formation by cultured rat phaeochromocytoma cells' Nature 258, 341-2, 1975

Todaro GJ, Rose TM, Spooner CE, Shoyab M and Plowman GD 'Cellular and viral ligands that interact with the EGF receptor' Semin Cancer Biol 1:257-63, 1990

Todd JH, Dowle C, Williams MR, Elston CW, Ellis IO, Hinton CP, Blamey RW and Haybittle JL 'Confirmation of a prognostic index in primary breast cancer' Br J Cancer 56:489-492, 1987

Toi M, Nakamura T, Mukaida H, Wada T, Osaki A, Yamada H, Toge T, Nimoto M and Hattori T 'Relationship between epidermal growth factor receptor status and various prognostic factors in human breast cancer' Cancer 65:1980-4, 1990

Toi M, Tominaga T, Osaki A and Toge T 'Role of epidermal growth factor expression in primary breast cancer: rsults of a biochemical study and an immunohistochemical study' Breast Cancer Res and Treat 29:51-58, 1994

Ullrich A, Coussens L, Hayflick JS, Dull TJ, Gray A, Tam AW, Lee J, Yarden Y, Liberman TA, Schlessinger J, Downward J, Mayes ELV, Whittle N, Waterfield MD and Seeburg PH 'Human epidermal growth factor receptor cDNA sequence and aberrant expression of the amplified gene in A431 epidermoid carcinoma cells' Nature 309:418-25, 1984

Ullrich A and Schlessinger J 'Signal transduction by receptors with tyrosine kinase activity' Cell 61:203-12, 1990

Umekita Y, Enokizono N, Sagara Y, Kuriwaki K, Takasaki T, Yoshida A and Yoshida H 'Immunohistochemical studies on TGFα in human breast cancer: their relationship to oestrogen receptor status, histological grade, mitogenic index and nodal status' Arch Pathol Hist 420:345-51, 1992

Valagussa P, Bonadonna G and Veronesi U 'Patterns of relapse and survival following radical mastectomy: Analysis of 716 consecutive patients' Cancer 41:1170-8, 1978

van Diest PJ, Weger DR and Lindholm J 'Reproducibility of subjective immunostaining of steriod receptors in breast cancer' Analytical and Quantitative Cytology and Histology 18(5): 351-4, 1996

Velu TJ, Vass WC, Lowy DR and Beguinot L 'Functional heterogeneity of the proto-oncogene tyrosine kinases: the C terminus of the human epidermal growth factor receptor facilitates cell proliferation' Mol Cell Biol 9:1772-1778, 1989

Vickers PJ, Dickson RB, Shoemaker R and Cowan K 'A multidrug resistant MCF7 human breast cancer cell line which exhibits cross-resistance to antioestrogens and hormone independent tumour growth in vivo' Molec Endocrinol 2:886-892, 1988

Vondherhaar B 'Local effect of EGF, α-TGF and EGF-like growth factors on lobuloalveolar development of the mouse mammary gland in vivo' J Cell Physiol 132:581-4, 1987

Vracko R 'Basal lamina scaffold-anatomy and significance for maintenance of orderly tissue structure' Am J Pathol. 77: 314-338, 1974

Wada T, Qian X and Greene MI 'Intermolecular association of $p185^{neu}$ protein and EGF receptor modulates EGF receptor function' Cell 61: 1339-1347, 1990.

Walker KJ, Price-Thomas JM, Candlish W and Nicholson RI 'Influence of the anti-oestrogen tamoxifen on normal breast tissue' Br J Cancer 64:764-8, 1991

Walker KJ, McClelland RA, Candlish W, Blamey RW and Nicholson RI 'Heterogeneity of oestrogen receptor expression in normal and malignan breast tissue' Eur J Cancer 28:34-7, 1992

Walker RA and Camplejohn RS 'DNA flow cytometry of human breast carcinomas and its relationship to transferrin and epidermal growth factor receptors' J. Pathol 150: 37-42, 1986

Walker RA and Dearing SJ 'Transforming growth factor $beta_1$in ductal carcinoma in situ and invasive carcinomas of the breast' European Journal of Cancer 28: 641-644, 1992

Weaver VM, Howlett AR, Langton-Webster B, Petersen OW and Bissell MJ 'The development of a functionally relevant cell culture model of progressive human breast cancer' Sem Cancer Biol 6:175-84, 1995

Weidner N and Gasparini G 'Determination of epidermal growth factor receptor provides additional prognostic information to measuring tumour angiogenesis in breast carcinoma patients' Breast Cancer Res Treat 29:97-107, 1994

Weiner DB, Liu J, Cohen JA, Williams WV and Greene MI 'A point mutation in the neu oncogene mimics ligand induction of aggregation' Nature 339:230-1, 1989

Wellings SR. 'Development of human breast cancer' Adv. Cancer. Res. 31:287-314, 1980

Wells A, Welsh JB, Lazar CS, Wiley HS, Gill GN and Rosenfeld MG 'Ligand induced transformation by a noninternalising epidermal growth factor receptor' Science 247:962-4, 1990

Welsh DR 'Comparison of tumour cell invasion assay: human amnion versus reconstituted basement membrane barriers' Invasion Metastasis 9: 278-297, 1989

Whelan T, Clark R, Roberts R, Levine M and Foster G 'Ipsilateral breast tumor recurrence postlumpectomy is predictive of subsequent mortality: results from a randomized trial. Investigators of the Ontario Clinical Oncology Group' Int J Radiation Oncology, Biology, Physics. 30(1):11-6, 1994

White JG, Amos WB and Fordham M 'An evaluation of confocal versus conventional imaging of biological structures by fluorescence light microscopy' J of Cell Biol., 105: 41-48, 1987

WHO 'Histologic typing of breast tumours' Geneve, Switzerland: WHO 1968

Wicha MS, Liotta LA, Garbisa S and Kidwell WR 'Basement membrane collagen requirements for attachment and growth of mammary epithelium' Exp Cell Res 124:181-90, 1980

Wiley LM, Adamson ED and Tsark EC 'Epidermal growth factor receptor function in early mammalian development' BioEssays 17:839-46, 1995

Willner J, Kiricuta IC and Kolbl O 'Locoregional recurrence of breast cancer following mastectomy: always a fatal event? Results of univariate and multivariate analysis' Int J Radiation Oncology, Biology, Physics. 37(4):853-63, 1997

Wilson T (Ed) 'Confocal Microscopy' Academic Press, 1990

Wolman SR, Smith HS, Stampfer M and Hackett AJ 'Growth of diploid cells from breast cancers' Cancer genetics and cryogenetics 16:49-64, 1985

Wrann MM and Fox CF 'Identification of epidermal growth factor receptors in a hyperproducing epidermal carcinoma cell line' J Biol Chem 254:8083-6, 1979

Wright C, Nicholson S, Angus B, Sainsbury JRC, Farndon JR, Cairns J, Harris AL and Horne CHW 'Relationship between c-erbB-2 protein product expression and response to endocrine therapy in advanced breast cancer' Br J Cancer 65:118-121, 1992

Xie H, Turner T, Wang MH, Singh RK, Siegal GP and Wells A 'In-vitro invasiveness of the du-145 human prostate carcinoma cells is modulated by EGF receptor mediated signals' Clinical and Experimental Metastasis 13: 407-419, 1995

Xu YH, Ishii S, Clark AJ, Sullivan M, Wilson RK, Ma DP, Roe BA, Merlino GT and Pastan I 'Human epidermal growth factor receptor cDNA is homologous to a variety of RNA's overproduced in A431 carcinoma cells' Nature 309:806-10, 1984

Yanai S, Sugiyama Y, Iga T, Fuwa T and Hanano M 'Kinetic analysis of the downregulation of epidermal growth factor receptors in rats in-vivo' Am J Physiol 258:C593-C598, 1990

Yarden Y and Schlessinger J 'Epidermal growth factor induces rapid, reversible aggregation of the purified epidermal growth factor receptor' Biochemistry 26:1443-51, 1987

Yee C and Shiu RPC 'Degradation of endothelial basement membrane by metastatic melanoma cells' Cancer Res 46: 1835-1839, 1986

Yoshida K, Tsujino T, Yasui W, Kameda T, Sano T, Nakayama H, Toge T and Tahara E 'Induction of growth factor receptor and metalloproteinase genes by epidermal growth factor and/or transforming growth factoe-alpha in human gastric carcinoma cell line MKN-28' Japanese Journal of Cancer Research 81:793-8, 1990

Zhang L, Chang CJ, Bacus SS and Hung MC 'Suppressed transformation and induced differentiation of HER-2/neu-overexpressing breast cancer cells by emodin' Cancer Research 55(17):3890-6, 1995 Sep 1.

APPENDICES

APPENDIX 1 Measurement studies

Table 1 : Ligand binding - raw data/tumours
Table 2 : Results from the LIGAND program/tumours
Table 3 : Results from the LIGAND program/placenta
Table 4 : Ligand binding - raw data/cell lines/run 1
Table 5 : Ligand binding - raw data/cell lines/run 2
Table 6 : Results from the LIGAND program/cell lines
Table 7 : Results from radioimmunohistochemistry/cell lines

Table 1 : Ligand binding - raw data/tumours
Results for series 1

tube	counts added	t1	t2	t3	t5	t7	t8	t9	t10	t11	t12	t14
1	22761	454	918	496	481	331	285	338	384	262	547	349
2	46086	991	1538	1039	940	654	579	611	752	878	1170	954
3	92191	1814	1791	1999	1672	1138	995	1453	904	1658	2091	1810
4	118945	1952	2164	2639	2225	965	1149	1067	1442	2140	2707	2047
5	136940	2963	3016	3284	2770	1186	765λ	895λ	1494	2660	3660	2567
6	188171	3626	4462	4574	3712	2375	1642	2096	1931	3426	4170	3472
7	347635	4950	5277	8731	6409	3521	2518	6787	6833	7733	8909	7301
8	477498	8144	6787	8679	5694	4401	7484	4257	7591	9649	11728	8497
9	136940	1358	2014	1753	1788	1557	871	1730	2430	2401	2793	3043
10	136940	1633	2147	2172	2502	1863	861	1016	2018	3137	3335	2498

key to table

tube : assay tube number
counts added : counts produced by the I^{125}-EGF added (per minute)
t(n) : radioactive counts per minute for tumour specimen (n)
'λ' : pipetting error with half volume of I^{125}-EGF added

Table 1 (cont.): Ligand binding - raw data/tumours
Results for series 2

tube	counts added	t4	t6	t13	t15	t16	t17	t18	t19	t20	t21	t22	t23
1	24639	662	1007	522	563	1152	497	436	575	1715	732	658	626
2	46347	1125	1143	927	1209	2514	1237	927	878	2019	1072	1086	1127
3	94966	2258	2214	1415	2021	4023	1867	1567	1688	3640	1955	1963	1977
4	110849	2552	3021	1985	2521	3936	2537	2231	2204	4970	2433	2480	2543
5	141607	3617	4343	2535	2954	4305	-	2619	2608	4487	2713	2944	4697
6	190406	5090	10573κ	3022	4303	5737	7651	4014	4279	5202	3798	3669	6211
7	338783	7816	4423λ	5192	7500	8166	7744	6982	7552	9267	6273	6026	13690
8	479152	11136	15268	8514	9806	10139	10623	9436	10319	12993	7922	7978	10672
9	141607	3524	3356	2333	2991	2723	3268	2815	2729	2692	2171	2135	2861
10	141607	-	3654	-	2648	2675	3195	2769	3145	2868	2257	2206	2619

key to table

tube : assay tube number
counts added : counts produced by the I^{125}-EGF added (per minute)
t(n) : radioactive counts per minute for tumour specimen (n)
'-' : indicates this data was unavailable for technical reasons
'κ' : pipetting error with double volume of I^{125}-EGF added
'λ' : pipetting error with half volume of I^{125}-EGF added

Table 1 (cont.): Ligand binding - raw data/tumours
Results for series 3

tube	counts added	t24	t25	t26	t27	t28	t29	t30
1	24888	2252	1167	862	-	-	338	864
2	46871	3279	1887	1595	1847	-	1576	1519
3	90863	6453	3315	3172	-	3078	2846	3244
4	109172	7409	4126	3362	4008	3677	3206	3752
5	136794	-	-	-	6733	4570	4335	4764
6	176568	8981	4478	4282	7626	5793	-	5918
7	314938	-	5588	5647	-	10524	-	10077
8	418539	-	-	9980	15738	13609	12136	14678
9	122524	3982	4222	3982	4831	3342	3736	4459
10	122524	4153	3912	4153	4518	3516	3790	4218

key to table

tube : assay tube number
counts added : counts produced by the I^{125}-EGF added (per minute)
t(n) : radioactive counts per minute for tumour specimen (n)
'-' : indicates this data was unavailable for technical reasons

Table 1 (cont.): Ligand binding - raw data/tumours
Results for series 3 (cont.)

tube	counts added	t31	t32	t33	t34	t35	t36	t37
1	24888	969	806	731	856	872	723	619
2	46871	1666	1573	1345	1638	1593	1410	1414
3	90863	3097	2908	2919	3029	3134	2554	2752
4	109172	3834	3523	3187	3474	3734	3163	3039
5	136794	4800	4680	4083	4409	4785	4117	3257
6	176568	6281	5737	5262	6016	5582	5372	6226
7	314938	11317	10333	10028	10733	10343	10503	10968
8	418539	15282	13154	10670	14178	13968	14115	14237
9	122524	4297	4248	3384	4047	4541	3860	4660
10	122524	4456	4108	3816	3804	3929	4152	4304

key to table

tube : assay tube number
counts added : counts produced by the I^{125}-EGF added (per minute)
t(n) : radioactive counts per minute for tumour specimen (n)

Table 1 (cont.): Ligand binding - raw data/tumours
Results for series 4

tube	counts added	t38	t39	t40	t41	t42	t43	t44
1	57705	1345	2049	1629	1523	1737	2429	1509
2	120466	2408	3366	3146	2835	3319	4941	2460
3	223197	3844	6261	5862	4905	5551	8453	4122
4	276685	5126	7495	6987	7208	6211	9803	5705
5	325085	5956	8355	9414	7567	8072	12154	7184
6	445626	7479	10197	11101	8647	9751	15080	8296
7	805755	13714	15799	22911	14704	15028	26946	14008
8	1076860	19086	22343	27744	20532	20034	42253	20465
9	323957	5338	5575	7718	5520	6667	6497	6339
10	323957	5070	5402	6648	6630	5800	6865	6032

key to table

tube : assay tube number
counts added : counts produced by the I^{125}-EGF added (per minute)
t(n) : radioactive counts per minute for tumour specimen (n)

Table 1 (cont.): Ligand binding - raw data/tumours
Results for series 4 (cont.)

tube	counts added	t45	t46	t47	t48	t49	t50
1	57705	1072	2085	1793	1460	1074	1731
2	120466	1809	4040	2715	2630	2187	2864
3	223197	3939	5461	5463	5034	4204	4748
4	276685	5430	7630	6457	5719	4994	5769
5	325085	8333	11852	9336	7440	5709	8500
6	445626	6967	11807	10256	10606	8608	8423
7	805755	11804	17877	19648	14901	12204	16691
8	1076860	26396	32599	37688	20499	19791	22883
9	323957	5460	6318	7699	5495	5501	6803
10	323957	5246	7127	7949	4790	6245	5700

key to table

tube : assay tube number
counts added : counts produced by the I^{125}-EGF added (per minute)
t(n) : radioactive counts per minute for tumour specimen (n)

Table 2 : Results from the LIGAND program/tumours

tumour	protein	R1	Receptor	tumour	protein	R1	Receptor	tumour	protein	R1	Receptor
t1	320	1.81E-11	136	t18	-	-	-	t35	795	-	-
t2	320	3.08E-12	23	t19	-	-	-	t36	773	-	-
t3	880	-	-	t20	7.44E-12	14	14	t37	1545	-	-
t4	720	-	-	t21	2.25E-12	5	5	t38	195	7.45E-13	9.2
t5	315	4.26E-12	32	t22	4.00E-12	11	11	t39	614	8.24E-12	32.2
t6	1260	-	-	t23	-	-	-	t40	605	-	-
t7	1350	-	-	t24	3.20E-14	79	79	t41	703	4.23E-12	14.4
t8	1064	-	-	t25	1.22E-12	3.4	3.4	t42	649	4.05E-12	15.0
t9	582	-	-	t26	975	-	-	t43	905	1.78E-10	472
t10	1714	-	-	t27	1081	-	-	t44	450	7E-13	3.7
t11	1227	-	-	t28	510	-	-	t45	750	5E-13	1.6
t12	1045	-	-	t29	655	-	-	t46	477	1.45E-11	73
t13	86	-	-	t30	700	-	-	t47	818	-	-
t14	855	-	-	t31	1209	-	-	t48	817	1.25E-11	36.7
t15	218	-	-	t32	491	-	-	t49	355	-	-
t16	800	1.03E-11	31	t33	668	-	-	t50	625	9E-13	3.5
t17	1227	-	-	t34	1400	-	-				

key to table

protein : concentration of membrane protein (μg/ml)
R1 : number of binding sites present determined by the LIGAND program (moles/l)
Receptor : Concentration of EGF receptor (fmol/mg)
'-' : The LIGAND program was unable to fit a curve to the data, interpreted as no measureable receptor

Table 3 : Results from the LIGAND program/placenta

	series 1		series 2		series 3		series 4	
tube	counts	p	counts	p	counts	p	counts	p
1	22761	10763	24639	12378	24888	11150	57705	22631
2	46086	21370	46347	19673	46871	20181	120466	38167
3	92191	26143	94966	28804	90863	25856	223197	63193
4	118945	34269	110849	37635	109172	19808	276685	86043
5	136940	35821	141607	38251	136794	35014	325085	73250
6	188171	47837	190406	53019	176568	53382	445626	98851
7	347635	71941	338783	72078	314938	37936	805755	145974
8	477498	81252	479152	88725	418539	73394	1076860	202353
9	136940	3216	141607	3156	122524	2291	323957	12137
10	136940	3520	141607	2785	122524	3962	323957	10187
protein	constant		constant		constant		constant	
R1	2.59E-10		3.02E-10		2.21E-10		4.06E-10	
%CV	18		15		31		20	
factor	1.09		1.04		1.28		0.69	

key to table

series (n) : indicates the tumour series, as per the tables 1 to 6
counts : counts produced by the I^{125}-EGF added (per minute)
p : counts from assay tube per minute
protein : membrane protein concentration was constant for the placental standard
R1 : number of binding sites present determined by the LIGAND program (moles/l)
%CV : %CV for the R1 value, determined by the LIGAND program
factor : standardising factor for each tumour series

Table 4 : Ligand binding - raw data/cell lines/run 1

		CELL LINES									
tube	counts added	SKBR3	MDA-MB-361	MDA-MB-453	MDA-MB-231	MCF7	MCF7-ADR	ZR75	ZR75	BT20	BT474
1	20972	5381	557	682	3197	480	1170	560	752	7179	4805
2	41616	8202	834	1338	5386	979	2643	1212	1115	10753	6579
3	82867	15806	1467	2095	9869	1625	3757	2038	2101	21459	15078
4	99054	17959	1545	2307	12030	1436	4961	1960	2480	25679	12762
5	126033	46883	2162	2533	13643	1629	6078	3180	3395	30070	15914
6	176797	21900	3024	3053	22130	3052	6871	2715	4207	33236	16130
7	324522	24556	5377	4847	34555	3840	11781	4299	6999	46096	21610
8	426619	31547	7391	7408	29083	5073	14877	5223	7898	85121	23836
9	137259	2071	1995	2047	2713	2204	1543	1592	2055	2703	2567
10	137259	2205	2132	1753	2647	-	1917	1548	2327	2614	2722

key to table

tube : assay tube number
counts added : counts produced by the I^{125}-EGF added (per minute)
CELL LINES : radioactive counts per minute for cell line indicated
'-' : indicates this data was unavailable for technical reasons

Table 5 : Ligand binding - raw data/cell lines/run 2

tube	counts added	CELL LINES SKBR3	MDA-MB-361	MDA-MB-453	MDA-MB-231	MCF7	MCF7-ADR	ZR75	ZR75	BT20	BT474
1	24142	7380	754	518	1944	528	1072	675	799	6403	604
2	49905	13660	946	1582	3252	1026	1664	1241	1694	10212	4238
3	90328	7110	2487	3288	4734	2154	2943	2527	1844	19367	5310
4	110170	19286	3112	3540	5310	3355	3631	3354	2629	21432	8000
5	126585	20600	3900	4217	5451	4475	5267	3195	3192	22735	7378
6	167483	18621	3782	5002	4637	4888	5517	4649	4417	25551	9702
7	307996	24442	7955	7820	10747	10189	10535	8980	7857	41495	9409
8	421361	20375	10182	10795	17153	10746	13872	12098	9363	40479	13318
9	113528	2516	2333	2563	2625	2918	2485	2577	1860	2949	2406
10	113528	2414	2943	2744	2496	2848	2264	2208	2215	2546	2720

key to table

tube : assay tube number
counts added : counts produced by the I^{125}-EGF added (per minute)
CELL LINES : radioactive counts per minute for cell line indicated

Table 6 : Results from the LIGAND program/cell lines

	protein		R1		Receptor		
cell line	Run 1	Run 2	Run 1	Run 2	Run 1	Run 2	combined
BT20	560	134	5.46 E-10	2.3 E-10	2340	4119	2628
BT474	440	114	9.40 E-11	5.1 E-11	513	1074	788
MDA-MB-231	420	420	2.63 E-10	1.5 E-11	1503	429	685
MCF7-ADR	460	92	7.45 E-11	7.9 E-11	389	2061	594
SKBR3	490	490	1.37 E-10	8.6 E-11	671	421	524
MCF7	780	780	1.76 E-12	1.0 E-15	5	-	29
MDA-MB-453	716	716	6.12 E-12	1.0 E-15	21	-	28
ZR75	1215	1215	9.80 E-12	6.17 E-12	19	12	25
MDA-MB-361	200	50	9.20 E-13	1.1 E-15	11	-	-

key to table

cell line : cell line studied
protein : concentration of membrane protein (μg/ml)
R1 : number of binding sites present determined by the LIGAND program (moles/l)
Receptor : Concentration of EGF receptor (fmol/mg of membrane protein)
Run : assay run number
combined : results for the combined analysis of run 1 and 2 data using the LIGAND program
'-' : The LIGAND program was unable to fit a curve to the data, interpreted as no measureable receptor

Table 7 : Results from Radioimmunohistochemistry/cell lines

cell line	test 1	test 2	control	exposure	g/mm2/h
BT20	9.12 E2	1.19 E3	5.2 E1	4	1753
BT474	6.91 E3	5.93 E3	1.3 E2	96	475
MDA-MB-231	1.36 E4	1.03 E4	1.35 E2	96	901
SKBR3	9.1 E2	1.02 E3	1.08 E2	24	231
MCF7	1.29 E3	1.37 E3	9.26 E2	96	-40
MDA-MB-453	1.24 E3	1.98 E3	6.8 E1	96	114
ZR75	2.35 E3	1.59 E3	3.13 E2	96	104
MDA-MB-361	1.24 E3	1.73 E3	2.13 E2	96	82
MCF7-ADR	-	-	-	-	-

Key to table

test 1 / test 2 : refer to the duplicate 'hot' sections - see study protocol

control : refers to the section treated with excess cold antibody

exposure : the duration, in hours, for which the radiographic emulsion was exposed

$g/mm^2/h$: silver grain counts per unit area per hour exposure

For each tumour cell line the area studied was $0.135mm^2$ (for the control sections the area assayed was $0.0675mm^2$).

The MCF7-ADR line was not studied using radioimmunohistochemistry.

APPENDIX 2 RADIOIMMUNOHISTOCHEMISTRY

Table 1: EGF receptor - relationship to prognostic factors

Case no	Path no	Age	EGFr gr	EGFr-n	size	T stage	n samp	n pos	n status	n group	ER-lig	ER-ihc	ER status	Grade
402957	9105824	61	0.00	0.000	20	2	14	0	0	1	71	200	1	2
503410	8410692	72	0.00	0.000	20	2	2	0	0	1	*	*	*	*
967906	8903674	61	0.00	0.000	20	2	7	7	1	3	*	*	*	3
954504	8901639	69	0.00	0.000	70	3	0	*	*	*	*	*	*	3
121385	8907716	59	0.00	0.000	20	2	4	0	0	1	183	*	1	2
063942	9009312	49	0.00	0.000	13	1	14	1	1	2	0	80	1	2
417929	9301463	63	0.00	0.000	12	1	10	0	0	1	104	152	1	3
446821	8701651	39	0.00	0.000	50	2	4	3	1	2	144	*	1	3
577412	9012098	58	0.00	0.000	50	2	0	*	*	*	0	0	0	2
140355	9204491	68	0.00	0.000	25	2	24	0	0	1	128	100	1	3
473196	8706410	62	0.00	0.000	10	1	3	0	0	1	202	*	1	2
342793	9204364	78	0.00	0.000	45	2	10	2	1	2	53	104	1	1
936787	8700804	59	0.00	0.000	25	2	2	1	1	2	0	*	0	3
288970	8700790	53	0.00	0.000	20	2	2	0	0	1	37	*	1	2
935171	8612150	69	0.00	0.000	105	3	5	5	1	3	*	*	*	2
702498	8502975	51	0.00	0.000	6	1	7	0	0	1	*	*	*	2
403133	9208764	76	0.04	0.003	30	2	9	5	1	3	26	*	1	3
968143	8904573	38	0.04	0.003	15	1	5	0	0	1	140	*	1	2
780821	9106709	60	0.06	0.004	32	2	10	10	1	3	*	*	*	3
206003	9300806	80	0.04	0.004	15	1	6	0	0	1	500	210	1	1
279044	8804484	67	0.07	0.007	25	2	3	0	0	1	28	*	1	1
515053	8703209	48	0.07	0.007	15	1	5	0	0	1	0	*	*	3
061145	9101093	77	0.10	0.008	20	2	4	0	0	1	500	*	1	2
503960	8805065	47	0.08	0.008	25	2	1	0	0	1	*	*	*	3
642261	8901273	62	0.10	0.009	15	1	6	0	0	1	49	*	1	2
402586	8712411	59	0.11	0.009	65	3	3	0	0	1	0	*	0	3
846528	8811908	63	0.12	0.010	20	2	9	0	0	1	*	*	*	2

Key is at table end

Table 1 (cont.): EGF receptor - relationship to prognostic factors

Case no	Path no	Age	EGFr gr	EGFr-n	size	T stage	n samp	n pos	n status	n group	ER-lig	ER-ihc	ER status	Grade
900137	8912026	53	0.17	0.011	60	3	10	2	1	2	35	*	1	2
974540	8812571	46	0.13	0.011	60	3	2	1	1	2	0	*	*	1
115372	8908009	75	0.14	0.012	20	2	1	0	0	1	71	*	1	2
708919	9302281	78	0.21	0.013	15	1	15	0	0	1	500	115	1	3
427023	9303058	62	0.21	0.013	12	1	12	5	1	3	84	170	1	2
297764	9200818	66	0.14	0.015	40	2	4	4	1	3	141	220	1	2
756189	9102368	60	0.25	0.015	40	2	12	0	0	1	38	160	1	2
954464	8902820	64	0.18	0.016	14	1	3	0	0	1	*	*	*	3
954415	8901286	65	0.18	0.016	20	2	0	*	*	*	*	*	*	3
239049	9208935	67	0.17	0.016	28	2	9	0	0	1	0	*	0	3
412085	9301824	68	0.26	0.017	60	3	1	1	1	2	210	90	1	2
665793	8905187	75	0.20	0.017	15	1	5	0	0	1	*	*	*	1
525216	9111426	53	0.19	0.018	25	2	5	2	1	2	29	120	1	1
748124	8505685	81	0.21	0.018	15	1	4	1	1	2	0	*	0	2
626866	9210338	68	0.19	0.018	20	2	10	3	1	2	500	170	1	2
377440	9207829	45	0.21	0.020	25	2	7	0	0	1	*	147	1	3
882081	8900595	68	0.23	0.020	50	2	*	*	*	*	*	*	*	3
391304	9204359	60	0.22	0.021	10	1	9	0	0	1	0	180	1	2
749619	9007679	71	0.23	0.021	30	2	0	*	*	*	500	*	1	1
116312	8806919	69	0.27	0.022	20	2	6	0	0	1	24	*	1	1
427751	9302680	44	0.35	0.022	20	2	12	0	0	1	106	120	1	2
103120	9002372	76	0.25	0.023	27	2	7	0	0	1	500	150	1	2
926342	8901930	53	0.28	0.023	15	1	4	0	0	1	*	*	*	1
047139	8704642	64	0.23	0.023	60	3	7	5	1	3	0	*	0	3
372967	9210524	61	0.24	0.023	15	1	8	6	1	3	0	0	0	3
692456	9200946	75	0.26	0.024	25	2	10	2	1	2	75	160	1	2
237782	8500080	57	0.28	0.025	45	2	5	1	1	2	87	184	1	1

Key is at table end

Table 1 (cont.): EGF receptor - relationship to prognostic factors

Case no	Path no	Age	EGFr gr	EGFr-n	size	T stage	n samp	n pos	n status	n group	ER-lig	ER-ihc	ER status	Grade
141677	9204266	65	0.27	0.025	50	2	10	8	1	3	50	158	1	2
016957	9001778	74	0.28	0.026	22	2	1	1	1	2	500	190	1	3
392543	8810981	74	0.27	0.027	80	3	4	4	1	3	0	*	0	2
731085	9206294	46	0.25	0.027	15	1	7	0	0	1	89	110	1	2
341393	9204693	87	0.28	0.027	30	2	12	0	0	1	76	150	1	2
712147	8803255	79	0.34	0.028	40	2	5	3	1	2	0	*	0	2
482237	8808944	53	0.28	0.028	25	2	12	12	1	3	0	*	0	2
169212	8602990	66	0.26	0.028	50	2	6	2	1	2	*	*	*	2
394682	9208073	47	0.29	0.028	16	1	6	0	0	1	56	125	1	2
959904	8905543	75	0.34	0.029	15	1	*	*	*	*	*	*	*	3
301509	8510456	67	0.35	0.030	130	3	0	*	*	*	*	*	*	3
400125	8804357	69	0.37	0.030	25	2	0	*	*	*	0	*	0	2
492717	9206780	76	0.32	0.031	33	2	3	0	0	1	55	220	1	2
334114	8707122	51	0.29	0.031	70	3	3	0	0	1	*	*	*	3
914369	8507901	76	0.37	0.032	50	2	10	3	1	2	113	*	1	3
975760	9205835	54	0.33	0.032	18	1	0	*	*	*	500	250	1	3
734711	8810776	73	0.33	0.033	30	2	6	6	1	3	*	*	*	2
547219	9102868	66	0.42	0.033	35	2	7	1	1	2	0	*	0	2
972627	8901400	62	0.39	0.034	30	2	4	0	0	1	1	*	1	3
955883	8803098	67	0.42	0.034	20	2	7	2	1	2	48	*	1	3
071212	9307150	61	0.45	0.035	98	3	0	*	*	*	*	*	*	2
401134	8904199	68	0.41	0.035	50	2	6	4	1	3	0	*	0	3
030205	9307605	61	0.46	0.036	28	2	12	12	1	3	0	0	0	3
172658	8706956	48	0.33	0.036	50	2	3	0	0	1	28	*	*	3
670691	8804019	56	0.46	0.038	18	1	7	0	0	1	*	*	*	3
695492	8612132	70	0.35	0.038	50	2	5	5	1	3	48	*	*	3
242414	8709028	69	0.35	0.038	75	3	4	1	1	2	150	*	1	3

Key is at table end

Table 1 (cont.): EGF receptor - relationship to prognostic factors

Case no	Path no	Age	EGFr gr	EGFr-n	size	T stage	n samp	n pos	n status	n group	ER-lig	ER-ihc	ER status	Grade
985246	8904491	50	0.44	0.038	20	2	17	1	1	2	25	*	1	2
957751	8811491	59	0.40	0.040	20	2	1	1	1	2	*	*	*	2
942538	8705899	42	0.40	0.040	10	1	1	0	0	1	0	*	0	2
176347	9204926	63	0.38	0.041	20	2	14	4	1	3	0	1	0	3
682111	9110864	56	0.53	0.042	15	1	5	0	0	1	350	146	1	3
391118	8910696	49	0.69	0.043	35	2	5	0	0	1	27	65	1	1
488487	8707318	65	0.40	0.043	30	2	4	0	0	1	*	*	*	3
608624	8708928	45	0.40	0.043	15	1	2	1	1	2	*	*	*	2
092169	9012080	63	0.70	0.043	20	2	6	0	0	1	311	250	1	2
756625	9210214	55	0.45	0.044	45	2	6	6	1	3	114	120	1	3
969436	8809490	53	0.45	0.045	25	2	7	0	0	1	71	*	1	3
983783	8809315	45	0.45	0.045	20	1	4	3	1	2	*	*	*	3
947575	8712557	77	0.56	0.046	45	2	2	2	1	2	*	*	*	1
531568	8500959	85	0.53	0.047	25	2	5	0	0	1	1	160	1	2
694917	9210380	63	0.68	0.047	19	1	9	0	0	1	0	*	0	1
981886	8807087	77	0.58	0.047	35	2	6	0	0	1	0	*	0	3
937789	9300505	77	0.51	0.048	40	2	2	2	1	2	0	52	1	2
746393	8500344	57	0.54	0.048	20	2	5	0	0	1	65	*	1	2
931713	9010281	47	0.80	0.050	14	1	5	0	0	1	74	135	1	3
388415	8809266	48	0.50	0.050	25	2	8	0	0	1	0	*	0	2
968888	8810478	65	0.50	0.050	20	2	2	0	0	1	*	*	*	2
717092	9109185	70	0.75	0.052	30	2	8	0	0	1	*	*	*	2
420772	8900435	40	0.61	0.053	8	1	5	0	0	1	*	*	*	1
650081	8808858	67	0.53	0.053	60	3	8	0	0	1	1	*	1	3
549335	8811312	43	0.53	0.053	15	1	1	0	0	1	*	*	*	1
916106	9205324	65	0.49	0.053	20	2	2	0	0	1	82	179	1	1
367231	9208915	61	0.50	0.054	70	3	1	1	1	2	97	15	1	3

Key is at table end

Table 1 (cont.): EGF receptor - relationship to prognostic factors

Case no	Path no	Age	EGFr gr	EGFr-n	size	T stage	n samp	n pos	n status	n group	ER-lig	ER-ihc	ER status	Grade
914193	8803618	49	0.69	0.056	16	1	12	8	1	3	27	*	1	2
422893	8604556	48	0.53	0.057	60	3	*	*	*	*	*	*	*	2
381435	9207616	58	0.62	0.058	10	1	9	1	1	2	*	*	*	3
496319	9109186	81	0.85	0.059	30	2	5	0	0	1	*	*	*	1
967875	8901654	29	0.74	0.061	38	2	14	0	0	1	*	*	*	3
478491	9307059	46	0.81	0.063	15	1	8	0	0	1	51	150	1	2
895535	8611383	74	0.60	0.065	30	2	3	0	0	1	63	*	1	2
750766	8910542	49	1.07	0.066	80	3	8	6	1	3	38	100	1	3
790984	8801091	75	0.82	0.067	25	2	6	3	1	2	47	*	1	2
191633	9306938	74	0.88	0.068	36	2	0	*	*	*	133	125	1	2
827673	9206709	77	0.71	0.069	30	2	3	2	1	2	42	104	1	2
376041	9108758	77	1.00	0.069	50	2	7	7	1	3	0	*	0	1
791710	8408726	63	0.79	0.070	40	2	2	2	1	2	0	0	0	2
144425	9205708	66	1.02	0.071	15	1	3	1	1	2	35	*	1	1
733437	8709507	43	0.66	0.071	40	2	5	5	1	3	0	*	0	2
245821	8707189	61	0.66	0.071	20	2	0	*	*	*	33	*	1	1
970483	8806329	59	0.72	0.072	30	2	7	6	1	3	46	*	*	3
476899	8707305	65	0.68	0.074	25	2	*	*	*	*	194	*	*	2
945826	8709046	77	0.70	0.076	40	2	0	*	*	*	1	*	*	2
296663	9200108	47	0.83	0.078	*	*	18	0	0	1	0	0	0	3
123588	8803383	60	0.96	0.079	20	2	8	1	1	2	0	*	*	1
129222	8904201	69	0.91	0.079	50	2	3	3	1	2	0	*	0	3
416865	9300815	60	0.73	0.079	20	2	12	1	1	2	500	125	1	3
250346	9011029	71	1.09	0.086	*	*	5	5	1	3	500	240	1	1
254152	9007702	76	1.18	0.093	20	2	*	*	*	*	416	*	1	2
127664	9000718	53	1.06	0.097	25	2	6	0	0	1	0	0	0	3
270235	8801987	30	1.20	0.098	120	3	0	*	*	*	0	*	*	3

Key is at table end

Table 1 (cont.): EGF receptor - relationship to prognostic factors

Case no	Path no	Age	EGFr gr	EGFr-n	size	T stage	n samp	n pos	n status	n group	ER-lig	ER-ihc	ER status	Grade
957375	9209759	66	1.13	0.110	25	2	8	8	1	3	0	0	0	3
376067	8911374	48	1.24	0.113	30	2	3	1	1	2	*	135	1	1
103083	8912036	53	1.89	0.117	120	3	8	0	0	1	21	95	1	3
900341	8500671	51	1.34	0.119	45	2	6	1	1	2	0	105	1	2
285559	8901822	57	1.57	0.128	25	2	3	0	0	1	*	*	*	3
726541	8810300	70	1.30	0.130	15	1	9	0	0	1	0	*	0	3
505687	9204087	57	1.55	0.145	35	2	11	1	1	2	0	0	0	3
289929	8910001	53	2.36	0.146	40	2	4	0	0	1	0	0	0	3
502533	9102367	66	1.98	0.157	20	2	5	0	0	1	161	*	1	3
311799	8602751	64	1.46	0.158	70	3	0	*	*	*	*	*	*	3
278671	9208751	64	1.69	0.159	18	1	7	0	0	1	147	135	1	3
437495	8808105	55	1.68	0.168	30	2	13	13	1	3	0	*	0	3
449799	9000072	70	1.98	0.180	50	2	10	3	1	2	151	160	1	2
501535	8808719	40	1.89	0.189	*	*	9	5	1	3	*	*	*	3
357863	8709016	52	1.75	0.190	25	2	*	*	*	*	0	*	*	3
287717	9111148	55	2.07	0.194	40	2	5	0	0	1	0	12	0	3
748251	8909985	47	2.60	0.201	22	2	3	2	1	2	0	*	0	3
310045	8411106	48	2.42	0.215	20	2	3	0	0	1	0	0	0	3
563869	9009463	52	2.94	0.233	50	2	10	0	0	1	*	0	0	3
217915	9107544	83	2.62	0.239	35	2	8	0	0	1	*	0	0	3
751548	8801636	38	2.92	0.239	30	2	4	1	1	2	0	*	0	2
359382	8410983	54	2.70	0.239	*	*	3	3	1	2	0	0	0	3
514843	8906139	47	2.81	0.243	40	2	7	0	0	1	0	*	0	3
431930	9303085	84	4.07	0.260	19	1	9	7	1	3	0	0	0	3
757968	9102372	52	3.40	0.269	110	3	6	5	1	3	36	145	1	3
114211	8503395	52	3.06	0.271	30	2	1	1	1	2	*	0	0	3
873241	8910541	58	3.16	0.288	50	2	4	1	1	2	0	9	0	3

Key is at table end

Table 1 (cont.): EGF receptor - relationship to prognostic factors

Case no	Path no	Age	EGFr gr	EGFr-n	size	T stage	n samp	n pos	n status	n group	ER-lig	ER-ihc	ER status	Grade
325597	8800584	62	3.60	0.294	28	2	10	0	0	1	*	*	*	3
464008	8411357	37	3.48	0.309	15	1	4	1	1	2	*	100	1	1
988067	9208355	77	3.29	0.319	12	1	0	*	*	*	0	0	0	3
710078	8711262	80	3.94	0.322	60	3	3	1	1	2	0	*	0	2
851459	8912138	63	3.56	0.325	70	3	9	0	0	1	0	0	0	3
943835	8708490	56	3.14	0.340	*	*	15	1	1	2	0	*	0	3
118398	9102613	59	4.48	0.346	15	1	3	0	0	1	0	*	0	3
924389	8603804	70	3.41	0.369	70	3	*	*	*	*	*	*	*	2
426984	9302669	50	6.34	0.404	25	2	5	0	0	1	0	0	0	3
679479	8910955	59	4.50	0.410	25	2	7	4	1	3	0	*	0	2
668765	9210381	53	7.13	0.455	15	1	12	0	0	1	0	0	0	2
173788	8806488	54	4.60	0.459	20	2	19	19	1	3	0	*	0	2
408344	9209218	50	6.80	0.470	15	1	9	0	0	1	0	*	0	3
948863	8801342	68	5.79	0.474	30	2	12	3	1	2	0	*	0	3
191673	9210533	67	7.51	0.479	30	2	2	0	0	1	0	*	0	3
119294	8709490	75	4.67	0.506	33	2	0	*	*	*	*	*	*	2
760905	9303049	40	9.07	0.578	19	1	5	2	1	2	0	0	0	3
095913	9100598	45	7.90	0.610	15	1	12	8	1	3	*	*	*	3
786964	8902838	59	7.36	0.636	40	2	1	1	1	2	*	*	*	3
970456	9207165	82	6.92	0.649	50	2	0	*	*	*	0	0	0	3
431937	9302797	48	10.25	0.654	19	1	14	0	0	1	333	135	1	1
265344	9003443	76	7.49	0.683	22	2	8	1	1	2	*	1	1	3
416483	9300785	48	6.53	0.709	40	2	5	1	1	2	30	0	1	2
972546	8810298	63	7.12	0.711	20	2	1	1	1	2	0	*	*	3
417137	9306747	60	9.35	0.722	50	2	6	3	1	2	0	0	0	3
280317	8705162	80	7.59	0.758	25	2	4	0	0	1	*	*	*	3
637246	9105576	72	10.14	0.802	35	2	2	1	1	2	*	0	0	3

Key is at table end

Table 1 (cont.): EGF receptor - relationship to prognostic factors

Case no	Path no	Age	EGFr gr	EGFr-n	size	T stage	n samp	n pos	n status	n group	ER-lig	ER-ihc	ER status	Grade
385714	8611361	64	16.20	1.754	25	2	5	0	0	1	0	*	0	2
467923	9110219	52	22.55	1.784	27	2	2	2	1	2	0	*	0	3
096740	9100823	74	29.24	2.314	50	2	0	*	*	*	*	0	0	2
587715	8800819	57	29.80	2.437	70	3	3	3	1	2	*	*	*	3
337071	9009311	77	89.40	7.074	45	2	8	0	0	1	0	0	0	2

Key to table

Case no : case recored number
Path no : pathology specimen number for that cancer
Age : patient age
EGF gr ; EGF receptor levels expressed as a percentage of A431 grain counts
EGFr-n ; EGF receptor levels expressed relative to the levels in normal breast (reduction mammoplasty specimens)
size : maximum tumour diameter in mm
T stage : size of tumour using TNM classification (no tumours classified as T4)
n samp : number of axillary lymph nodes sampled
n pos : number of axillary lymph nodes histologically positive for metastatic breast cancer
n status : 1 indicates axillary lymph node metastasis, 0 indicates no nodal involvement
n group : 1 indicates no nodal involvement, 2 indicates 1-3 nodes involved, 3 indicates 4 or more nodes involved
ER-lig : oestrogen receptor in femto-moles, determined by ligand binding
ER-ihc : oestrogen receptor levels determined by immunohistochemistry; 0 is negative, 1 is positive
ER status : oestrogen receptor expression, using the ER-lig and ER-ihc results (see Ch 7 sect. ii); 0 is negative, 1 is positive
Grade ; modified Bloom and Richardson tumour grade

Table 2: EGF receptor - outcome

Case no	Path no	Tamoxifen	Radio-T	Chemo-T	Follow up	Deaths	Distal-R	Local-R	DFS
402957	9105824	1	1	0	2246	1	*	*	*
503410	8410692	*	*	*	2253	2	*	*	*
967906	8903674	1	1	0	223	3	136	*	136
954504	8901639	*	*	*	187	3	15	*	15
121385	8907716	1	0	0	2691	1	*	*	*
063942	9009312	0	1	1	2143	1	*	*	*
417929	9301463	1	0	0	1651	1	*	*	*
446821	8701651	*	*	1	1623	3	1018	196	196
577412	9012098	1	0	0	1102	3	0	470	0
140355	9204491	1	0	0	1896	1	*	*	*
473196	8706410	1	1	0	3432	1	*	*	*
342793	9204364	1	0	0	1529	1	*	*	*
936787	8700804	*	*	*	513	3	491	371	371
288970	8700790	1	1	0	3782	1	*	*	*
935171	8612150	*	*	*	389	3	389	*	389
702498	8502975	*	*	*	0	1	*	*	*
403133	9208764	1	0	0	352	3	279	*	279
968143	8904573	*	1	*	1297	3	1297	386	386
780821	9106709	1	0	0	354	3	193	222	193
206003	9300806	1	0	0	1244	2	*	*	*
279044	8804484	1	0	0	3382	1	*	3235	3235
515053	8703209	0	1	1	2558	3	2254	2315	2254
061145	9101093	1	0	0	2214	1	*	*	*
503960	8805065	*	*	*	1374	3	1316	*	1316
642261	8901273	1	1	0	2969	1	*	*	*
402586	8712411	*	*	*	2804	3	1050	289	289
846528	8811908	1	1	0	3232	1	*	*	*

Key is at table end

Table 2 (cont.): EGF receptor - outcome

Case no	Path no	Tamoxifen	Radio-T	Chemo-T	Follow up	Deaths	Distal-R	Local-R	DFS
900137	8912026	1	0	0	2715	1	*	*	*
974540	8812571	0	1	1	2814	1	*	43	43
115372	8908009	1	0	0	2392	1	*	*	*
708919	9302281	1	0	0	1444	1	*	*	*
427023	9303058	1	0	0	1630	1	*	*	*
297764	9200818	1	0	0	1745	1	*	*	*
756189	9102368	1	0	0	2143	1	*	*	*
954464	8902820	1	0	0	3151	1	*	*	*
954415	8901286	1	0	0	2893	1	*	*	*
239049	9208935	1	1	0	374	3	211	*	211
412085	9301824	1	0	0	1556	1	*	*	*
665793	8905187	1	0	0	2837	1	*	*	*
525216	9111426	1	0	0	1994	1	*	*	*
748124	8505685	0	0	0	695	3	695	*	695
626866	9210338	1	1	0	1842	1	*	*	*
377440	9207829	1	0	0	1489	1	*	*	*
882081	8900595	*	*	*	317	3	317	*	317
391304	9204359	1	0	0	1934	1	*	*	*
749619	9007679	1	*	*	2340	2	*	*	*
116312	8806919	1	1	0	3268	1	*	*	*
427751	9302680	0	1	1	1079	1	*	*	*
103120	9002372	1	0	0	2349	1	*	*	*
926342	8901930	1	0	0	3034	1	*	*	*
047139	8704642	*	*	*	213	2	*	*	*
372967	9210524	1	1	0	1755	1	*	*	*
692456	9200946	1	0	0	2022	1	*	*	*
237782	8500080	0	1	0	1034	3	855	*	855

Key is at table end

Table 2 (cont.): EGF receptor - outcome

Case no	Path no	Tamoxifen	Radio-T	Chemo-T	Follow up	Deaths	Distal-R	Local-R	DFS
141677	9204266	1	1	0	649	3	481	*	481
016957	9001778	1	1	0	2702	1	*	*	*
392543	8810981	0	1	0	741	3	478	*	478
731085	9206294	1	1	0	1699	1	*	*	*
341393	9204693	1	0	0	1826	1	*	*	*
712147	8803255	1	1	0	627	3	627	514	514
482237	8808944	*	*	1	1275	3	534	353	353
169212	8602990	*	*	*	903	3	903	*	903
394682	9208073	1	0	0	1670	1	*	*	*
959904	8905543	1	0	0	2082	2	*	2002	2002
301509	8510456	1	1	0	595	3	443	*	443
400125	8804357	1	1	0	1933	3	1933	*	1933
492717	9206780	1	0	0	1423	1	*	*	*
334114	8707122	1	1	0	2183	3	1684	1684	1684
914369	8507901	1	0	0	2258	3	1640	*	1640
975760	9205835	1	1	0	716	3	493	*	493
734711	8810776	1	1	0	1122	2	*	*	*
547219	9102868	1	0	0	1896	3	1896	*	1896
972627	8901400	1	0	0	2644	1	2077	2077	2077
955883	8803098	1	1	0	3410	1	*	*	*
071212	9307150	1	1	0	869	1	*	*	*
401134	8904199	1	1	0	1672	3	1672	1491	1491
030205	9307605	1	*	*	190	3	190	92	92
172658	8706956	0	1	0	1051	3	809	*	809
670691	8804019	1	0	0	3221	1	*	*	*
695492	8612132	1	0	0	1211	3	1075	*	1075
242414	8709028	1	0	0	1242	3	777	716	716

Key is at table end

Table 2 (cont.): EGF receptor - outcome

Case no	Path no	Tamoxifen	Radio-T	Chemo-T	Follow up	Deaths	Distal-R	Local-R	DFS
985246	8904491	1	0	0	2885	1	*	*	*
957751	8811491	*	*	*	508	3	508	*	508
942538	8705899	1	1	0	839	1	*	*	*
176347	9204926	1	0	0	177	2	158	*	158
682111	9110864	1	1	0	1853	1	*	*	*
391118	8910696	1	0	0	2945	1	*	*	*
488487	8707318	*	*	*	1172	2	*	*	*
608624	8708928	0	1	1	2350	3	2269	2220	2220
092169	9012080	1	0	0	2340	1	*	*	*
756625	9210214	1	1	0	1797	1	12	*	12
969436	8809490	1	0	0	2916	1	*	504	504
983783	8809315	*	*	*	2324	3	2324	*	2324
947575	8712557	1	0	0	2160	3	2160	466	466
531568	8500959	1	0	0	1116	2	*	*	*
694917	9210380	1	1	0	1674	1	*	*	*
981886	8807087	0	0	0	2485	3	2485	*	2485
937789	9300505	1	0	0	1660	1	*	610	610
746393	8500344	0	1	0	4405	1	*	*	*
931713	9010281	1	1	0	1902	1	*	1848	1848
388415	8809266	1	0	0	1075	3	1012	1012	1012
968888	8810478	*	*	*	1902	3	1902	539	539
717092	9109185	1	0	0	2025	1	*	*	*
420772	8900435	0	1	1	3215	1	*	*	*
650081	8808858	1	0	0	2308	2	*	*	*
549335	8811312	*	*	*	1961	1	*	1575	1575
916106	9205324	1	0	0	1672	1	*	*	*
367231	9208915	1	1	0	1643	1	790	*	790

Key is at table end

Table 2 (cont.): EGF receptor - outcome

Case no	Path no	Tamoxifen	Radio-T	Chemo-T	Follow up	Deaths	Distal-R	Local-R	DFS
914193	8803618	1	1	0	3427	1	*	*	*
422893	8604556	*	*	*	645	2	292	*	292
381435	9207616	1	1	0	1840	1	*	*	*
496319	9109186	1	0	0	21	2	*	*	*
967875	8901654	0	0	1	2890	1	*	*	*
478491	9307059	0	1	1	1553	1	*	*	*
895535	8611383	0	0	0	3138	2	*	497	497
750766	8910542	1	1	0	865	3	366	*	366
790984	8801091	1	0	0	1095	1	*	*	*
191633	9306938	1	1	0	1477	1	*	*	*
827673	9206709	1	0	0	900	1	*	*	*
376041	9108758	1	0	0	975	3	861	*	861
791710	8408726	0	0	0	456	3	456	240	240
144425	9205708	1	0	0	1759	1	*	*	*
733437	8709507	0	0	1	1658	3	1613	761	761
245821	8707189	1	0	0	2349	2	*	*	*
970483	8806329	1	1	0	2662	3	2286	1921	1921
476899	8707305	1	0	0	89	3	4	*	4
945826	8709046	1	0	0	2006	2	*	*	*
296663	9200108	0	1	1	2053	1	*	*	*
123588	8803383	0	1	0	3264	1	*	*	*
129222	8904201	1	0	0	715	2	471	*	471
416865	9300815	1	0	0	1055	3	705	*	705
250346	9011029	1	0	0	642	2	*	*	*
254152	9007702	1	0	0	2228	1	*	*	*
127664	9000718	1	0	0	2668	1	*	*	*
270235	8801987	0	0	0	433	3	433	190	190

Key is at table end

Table 2 (cont.): EGF receptor - outcome

Case no	Path no	Tamoxifen	Radio-T	Chemo-T	Follow up	Deaths	Distal-R	Local-R	DFS
957375	9209759	1	1	0	419	3	70	*	70
376067	8911374	0	1	1	2206	3	2206	*	2206
103083	8912036	1	0	0	569	3	420	205	205
900341	8500671	0	0	1	4397	1	*	*	*
285559	8901822	1	1	0	2924	1	*	*	*
726541	8810300	1	0	0	3275	1	*	*	*
505687	9204087	1	0	0	1714	1	*	*	*
289929	8910001	1	1	0	1629	3	1353	892	892
502533	9102367	1	0	0	2277	1	*	*	*
311799	8602751	*	*	*	254	3	254	*	254
278671	9208751	1	0	0	1819	1	*	*	*
437495	8808105	*	1	*	600	3	600	255	255
449799	9000072	1	0	0	2826	1	*	*	*
501535	8808719	*	*	*	339	2	0	56	0
357863	8709016	0	0	1	3710	1	*	1230	1230
287717	9111148	1	*	*	279	3	279	*	279
748251	8909985	0	0	1	969	3	500	837	500
310045	8411106	0	0	1	72	2	*	*	*
563869	9009463	*	*	*	447	3	447	*	447
217915	9107544	1	0	0	1750	3	1750	*	1750
751548	8801636	0	0	1	2088	3	2088	379	379
359382	8410983	0	0	1	4445	1	*	*	*
514843	8906139	0	1	0	162	1	*	*	*
431930	9303085	1	0	0	954	1	950	823	823
757968	9102372	1	0	0	2226	3	1511	*	1511
114211	8503395	*	*	*	966	3	966	*	966
873241	8910541	1	0	0	633	3	633	*	633

Key is at table end

Table 2 (cont.): EGF receptor - outcome

Case no	Path no	Tamoxifen	Radio-T	Chemo-T	Follow up	Deaths	Distal-R	Local-R	DFS
402957	9105824	1	0	0	0	1	*	*	*
503410	8410692	*	*	1	1743	3	864	560	560
967906	8903674	1	0	0	744	3	442	*	442
954504	8901639	*	*	*	1387	3	1387	*	1387
121385	8907716	1	0	0	1937	2	*	*	*
063942	9009312	0	1	0	0	1	*	*	*
417929	9301463	1	0	0	1862	3	1862	378	378
446821	8701651	*	*	*	117	3	117	*	117
577412	9012098	0	0	1	1515	1	*	*	*
140355	9204491	1	1	0	1390	3	751	1056	751
473196	8706410	1	0	0	765	3	629	*	629
342793	9204364	1	1	0	2875	1	*	*	*
936787	8700804	1	0	0	1853	1	*	*	*
288970	8700790	0	0	1	1116	3	1116	*	1116
935171	8612150	1	*	*	504	1	*	*	*
702498	8502975	1	0	0	603	2	578	154	154
403133	9208764	0	1	1	853	3	663	*	663
968143	8904573	1	0	1	1828	3	1828	*	1828
780821	9106709	1	1	0	1231	3	1231	735	735
206003	9300806	1	0	0	330	3	298	*	298
279044	8804484	1	0	0	1658	1	*	*	*
515053	8703209	1	0	0	596	3	570	325	325
061145	9101093	0	0	1	568	3	568	187	187
503960	8805065	0	1	1	1531	3	0	*	0
642261	8901273	1	1	0	379	3	323	*	323
402586	8712411	*	*	*	1500	3	1132	1041	1041
846528	8811908	1	0	0	1668	3	1171	379	379

Key is at table end

Table 2 (cont.): EGF receptor - outcome

Case no	Path no	Tamoxifen	Radio-T	Chemo-T	Follow up	Deaths	Distal-R	Local-R	DFS
385714	8611361	0	0	0	3991	1	*	*	*
467923	9110219	1	0	0	2304	1	*	*	*
096740	9100823	1	0	0	307	3	245	*	245
587715	8800819	*	*	*	697	2	*	*	*
337071	9009311	1	1	0	419	3	419	334	334

Key to table

Case no : case recored number
Path no : pathology specimen number for that cancer
Tamoxifen : Received adjuvant Tamoxifen (1) or did not (0)
Radio-T : Received adjuvant radiotherapy (1) or did not (0)
Chemo-T : Received adjuvant chemotherapy(1) or did not (0)
Follow up : Duration (days) of follow up from date of surgery surgery
Deaths : Still alive (1), death from cause not directly related to breast cancer (2), and death that was breast cancer related (3)
Distal-R : Duration (days) from surgery until diagnosis of distal recurrence
Local-R : Duration (days) from surgery until diagnosis of local recurrence
DFS : Duration (days) from surgery until diagnosis of any recurrence (disease free survival)

Table 3 : EGF receptor - batches and controls

	Tumour batches						
date	14.04.94	18.04.94	29.04.94	05.06.95	06.06.95	07.06.95	08.06.95
A431 (control)	170.0	190.0	148.1	121.3	131.6	160.6	152.1
Path no.	9101093	9210380	8410692	8706410	8701651	8901639	8903674
"	9307059	9109185	8502975	8700804	8804484	8712411	8907716
"	8909985	9109186	8500080	8700790	8703209	8806919	8904573
"	9102613	9108758	8500959	8612150	8805065	8901930	8901273
"	9306747		8500344	8602990	8704642	8803255	8811908
"	9307605		8408726	8707122	8810981	8804357	8812571
"			8500671	8706956	8808944	8803098	8908009
"			8411106	8612132	8810776	8804019	8902820
"			8410983	8708928	8811491	8712557	8901286
"			8503395	8604556	8705899	8807087	8905187
"			8411357	8611383	8809490	8803618	8505685
"				8709507	8809315	8901654	8900595
"				8707189	8809266	8801091	8905543
"				8707305	8810478	8803383	8510456
"				8709046	8808858	8801987	8507901
"				8602751	8811312	8901822	8901400
"				8709016	8806329	8801636	8904199
"				8708490	8810300	8800584	8904491
"				8603804	8808105	8711262	8900435
"				8709490	8808719	8801342	8904201
"				8611361	8806488	8800819	8906139
"				8709028	8810298		8902838
"					8705162		

Key is at table end

Table 3 (cont.): EGF receptor - batches and controls

	Tumour batches						
Date	06.04.93	13.04.93	04.05.93	06.05.93	11.05.93	08.06.93	10.06.93
A431 (control)	166.0	144.1	140.0	135.3	121.0	206.0	212.2
Path no.	9105824	9012098	9204364	9204491	9200818	9009312	9106709
"	9102868	9007679	9208935	9300806	9206294	9301463	8912026
"	9110864	9002372	9111426	9210338	9204926	9208764	9102368
"	9011029	9001778	9207829	9210524	9205324	9303058	8910696
"	9007702	8910542	9200946	9204693	9208915	9301824	9012080
"	9102367	9000718	9204266	9208073	9300815	9302680	9010281
"	9009463	8911374	9300505	9206780	9100823	9303085	8912036
"	9102372	9000072	9207616	9205835		9210381	8910001
"	9009311	8910541	9200108	9210214		9210533	
"		8912138	9204087	9206709		9303049	
"		8910955	9208751	9209759		9302797	
"		9003443	9111148	9208355			
"			9207165				

Key to table

date : Date of radioimmunohistochemical assay of tumours

A431 (control) : grain counts for A431 cells in that batch (used to standardise tumour results)

path no. : path number of tumours in that batch

Table 4 : EGF receptor - normal breast

Specimen	grains normal	grains A431	%A431
143960C	12.70	160.0	160.0
159853C	16.34	212.2	212.2
170174C	12.50	190.0	190.0
170806C	10.90	160.0	160.0
174198C	15.52	160.0	160.0
175279C	14.50	160.0	160.0
175771C	15.40	160.0	160.0
178191C	8.75	160.0	160.0
179648C	10.03	190.0	190.0

Key to table

specimen : path no. of the reduction mammoplasty specimen

grains : radioimmunohistochemical grain counts for reduction mammoplasty tissue (normal) or A431 (control)

%A431 : reduction mammoplasty grains as a percent of the A431 grain count for that assay

APPENDIX 3 INVASION STUDIES

General key to tables

The data in the following tables was used to construct the plots presented in the text.
Tables are named after the figures they were used to construct. If more than one figure was produced from a table then the names of each of these tables are given
The keys at the foot of each table provide specific details for that particular assay.

Terms

Assay	: a series, usually 6, of inserts
Insert	: an individual filter assembly
Field	: a single optical field in an insert
*n*µm	: refers to a the % fluorescing pixels at a distance *n*µm from the filter
EGF	: nanograms Epidermal growth factor added above the Matrigel layer
tot p	: the sum of pixels in the optical sections above the filter
ratio	: expressed as sum of pixels at 15, 18 and 21µm divided by the sum of pixels at 0, 3 and 6µm
s{}	: prefix indicating the variable {} is standardised to give a range of 0-1
Filter	: cells below the filter were removed (wiped) or left (unwiped) prior to reading the assay

Table 1: Invasion using different chemoattractants / The effect of 1% FCS and 20ng EGF as chemoattractant

Assay	Insert	attractant	0 µm	5 µm	10 µm	15 µm	20 µm	25 µm	tot P
1	1	control	0.4	11.9	26.0	14.0	4.2	0.8	57.3
1	2	EGF	0.3	7.5	21.0	10.2	4.0	2.0	45.0
1	3	TGF	0.2	12.8	22.3	6.0	0.5	0.1	41.9
1	4	FGF	1.5	24.3	16.4	3.5	0.4	0.0	46.1
1	5	Serum	0.3	9.8	20.7	9.5	1.3	0.1	41.7
1	6	Con. Medium	0.1	8.4	12.8	3.8	0.9	0.2	26.2
2	1	control	0.1	1.7	16.5	18.4	5.9	0.6	43.2
2	2	EGF	0.2	6.8	13.6	5.5	1.6	0.5	28.2
2	3	TGF	1.0	17.5	16.9	3.1	0.1	0.0	38.6
2	4	FGF	1.2	18.5	18.0	3.8	0.4	0.1	42.0
2	5	Serum	4.5	16.4	9.8	1.9	0.2	0.0	32.8
2	6	Con. Medium	0.5	11.0	10.8	2.8	0.3	0.0	25.4
3	1	control	2.3	14.1	19.3	9.7	2.5	0.3	48.2
3	2	EGF	0.0	1.8	10.0	8.8	4.6	2.3	27.5
3	3	TGF	0.7	4.0	3.0	0.3	0.0	0.0	8.0
3	4	FGF	0.0	0.8	7.4	7.1	2.9	0.6	18.8
3	5	Serum	0.3	3.1	5.4	3.0	0.5	0.0	12.3
3	6	Con. Medium	0.1	1.4	2.4	0.6	0.1	0.1	4.7
4	1	control	0.0	6.0	3.1	2.7	1.5	0.2	13.5
4	2	EGF	0.0	2.2	5.0	2.0	0.6	0.2	10.0
4	3	TGF	0.2	1.4	1.8	0.3	0.0	0.0	3.7
4	4	FGF	0.2	2.8	4.2	2.7	1.3	0.4	11.6
4	5	Serum	0.7	0.4	0.5	0.4	0.1	0.0	2.1
4	6	Con. Medium	0.0	0.9	3.8	1.4	0.1	0.0	6.2

Key to table (see general key at beginning of Appendix 3)

attractant : Chemoattractant added above the Matrigel layer
Control : Culture medium with 1% foetal calf serum
EGF : Culture medium with 1% foetal calf serum + 20ng Epidermal growth factor per well
TGF : Culture medium with 1% foetal calf serum + 1ng Transforming growth factor β per well
FGF : Culture medium with 1% foetal calf serum + 10ng Acidic fibroblastgrowth factor per well
Serum : Culture medium with 10% foetal calf serum
Con. Medium : Fibroblast conditioned culture medium

Table 2: Determining a control for the invasion studies

Assay	Field	Control	1%s	10%s	EGF-1%s	EGF-10%s
1	1	0.6	0.6	3.7	3.6	4.6
1	2	0.2	0.3	0.5	0.8	3.1
1	3	0.0	0.5	2.4	1.3	1.2
1	4	0.2	0.6	2.5	2.4	2.5
1	5	0.1	0.5	0.9	1.7	1.9
2	1	0.4	0.2	1.1	1.5	1.2
2.	2	0.3	0.5	2.8	2.2	4.4
2	3	0.5	1.1	1.9	0.9	2.2
2	4	0.1	0.7	1.0	2.8	3.8
2	5	0.3	0.3	0.6	1.2	2.6

Key to table (see general key at beginning of Appendix 3)

Control : Culture medium only above Matrigel layer

1%s : Culture medium + 1% foetal calf serum above Matrigel layer

10%s : Culture medium + 10% foetal calf serum above Matrigel layer

EGF-1%s : Culture medium + 1% foetal calf serum + 20ng Epidermal growth factor above Matrigel layer

EGF-10%s : Culture medium + 10% foetal calf serum + 20ng Epidermal growth factor above Matrigel layer

Table 3: Determining the significance of the concentration gradient / Comparing the methods of assay interpretation

Insert	Field	EGF (ng)	0μm	3μm	6μm	9μm	12μm	15μm	18μm	21μm	ratio	s ratio	tot p
1	1	-10	40.1	38.9	28.7	19.5	12.8	8.6	5.6	3.4	0.163	1.000	157.6
1	2	-10	33.0	24.1	15.3	10.2	7.0	4.0	1.7	0.6	0.087	0.416	95.9
1	3	-10	34.3	26.6	17.7	11.4	7.8	5.3	3.1	1.2	0.122	1.684	107.4
1	4	-10	35.8	30.8	20.5	12.7	8.3	5.0	2.9	1.5	0.108	0.576	117.5
1	5	-10	28.2	26.3	17.9	10.9	6.3	3.7	1.9	0.9	0.090	0.437	96.1
2	1	0	43.3	38.2	26.1	10.6	5.8	2.6	0.9	0.2	0.034	0.014	127.7
2	2	0	50.0	36.6	22.7	12.4	6.6	3.2	1.2	0.4	0.044	0.087	133.1
2	3	0	37.7	34.0	28.8	13.9	7.1	3.5	1.4	0.6	0.055	0.169	127.0
2	4	0	27.5	28.6	14.5	7.2	3.5	1.6	0.5	0.2	0.033	0.000	83.6
2	5	0	45.7	35.9	21.7	11.6	6.2	2.8	1.2	0.3	0.042	0.069	125.4
3	1	30	20.3	16.2	10.2	6.1	3.6	1.9	0.9	0.4	0.069	0.275	59.6
3	2	30	15.1	11.9	7.5	4.3	2.4	1.3	0.6	0.2	0.061	0.216	43.6
3	3	30	35.6	28.9	18.0	9.9	5.4	2.9	1.5	0.7	0.062	0.223	102.9
3	4	30	30.6	24.9	16.1	9.0	4.8	2.6	1.4	0.7	0.066	0.253	90.1
3	5	30	21.1	15.4	8.6	4.5	2.3	1.1	0.5	0.3	0.042	0.073	53.8
4	1	50	25.4	17.4	9.7	4.9	2.7	1.5	0.6	0.2	0.044	0.086	62.4
4	2	50	19.1	18.6	12.8	7.4	3.9	2.2	1.3	0.7	0.083	0.387	66.0
4	3	50	24.6	19.2	11.8	6.2	3.6	2.1	1.2	0.5	0.068	0.273	69.2
4	4	50	15.5	13.2	8.6	4.6	2.3	1.2	0.6	0.3	0.056	0.181	46.3
4	5	50	16.7	13.2	8.2	4.4	2.6	1.8	1.2	0.7	0.097	0.493	48.8
5	1	70	27.2	21.9	13.3	7.3	4.0	2.0	1.1	0.6	0.059	0.204	77.4
5	2	70	23.7	24.6	18.3	11.8	7.1	4.0	1.9	0.8	0.101	0.520	92.2
5	3	70	18.9	16.0	9.5	5.1	2.9	1.4	0.6	0.2	0.050	0.130	54.6
5	4	70	18.2	19.0	13.6	8.5	5.1	3.2	2.1	1.3	0.130	0.744	71.0
5	5	70	16.1	16.4	10.6	5.8	2.8	1.3	0.5	0.3	0.049	0.123	53.8

Key to table (see general key at beginning of Appendix 3)

EGF (ng) : ng Epidermal growth factor added above Matrigel layer,
negative prefix indicates that the growth factor was added below the filter

Table 4: Determining the significance of the Matrigel meniscus

Insert	position	tot P	s tot p
1	-2	17.8	0.100
1	-1	70.8	1.000
1	0	12.0	0.000
1	1	51.9	0.679
1	2	70.7	0.998
2	-2	59.4	0.753
2	-1	68.0	1.000
2	0	56.2	0.661
2	1	33.2	0.000
2	2	44.7	0.330
3	-2	17.9	0.569
3	-1	20.6	0.757
3	0	14.2	0.313
3	1	9.7	0.000
3	2	24.1	1.000

Key to table (see general key at beginning of Appendix 3)

position : refers to the position across the meniscus, the values are distances to each side of the central point (0) from which readings were taken

Table 5: Altering the duration of the assay incubation

Days	0µm	5µm	10µm	15µm	20µm	25µm	tot p
4	13.3	12.9	7.6	4.0	1.9	0.9	40.6
4	10.3	9.2	5.0	2.2	1.0	0.4	28.1
4	14.3	11.0	4.5	1.8	0.6	0.3	32.5
7	13.2	9.3	5.6	3.9	2.6	1.7	36.3
7	22.2	15.6	7.7	3.7	2.2	1.4	52.8
7	17.4	14.0	8.8	4.9	2.5	1.3	48.9
10	19.3	15.4	9.0	5.2	2.7	1.1	52.7
10	14.9	11.0	5.8	2.7	1.3	0.4	36.1
10	19.6	14.9	8.8	4.9	3.0	1.3	52.5

Key to table (see general key at beginning of Appendix 3)

Days : duration of assay incubation in days

Table 6: Wiping the filter bottom improves assay accuracy / Reading the same confocal field / Reading the same optical section series

Insert	Filter	0µm	3µm	6µm	9µm	12µm	15µm	18µm	21µm	0µm/mean	tot p	tot p/mean
1	unwiped	31.0	24.5	19.8	15.8	12.6	7.8	5.0	3.4	1.191	119.9	1.232
1	unwiped	23.8	19.1	17.1	12.4	8.3	4.9	3.4	2.1	0.915	91.1	0.936
1	unwiped	26.7	20.6	16.4	13.0	9.1	6.0	3.5	2.2	1.026	97.5	1.002
1	unwiped	28.1	22.1	17.1	13.8	10.1	6.6	4.0	2.4	1.080	104.2	1.070
1	unwiped	20.5	16.6	13.3	9.5	6.4	3.9	2.4	1.4	0.789	74.0	0.760
2	wiped	13.2	13.4	11.8	9.3	6.9	4.9	3.3	2.2	1.015	65.0	1.170
2	wiped	13.6	12.4	10.5	7.9	5.6	3.9	2.5	1.6	1.046	58.0	1.044
2	wiped	12.8	11.6	9.4	6.8	4.9	3.4	2.0	1.2	0.985	52.1	0.938
2	wiped	12.5	11.1	8.6	6.3	4.3	2.9	1.9	1.1	0.962	48.7	0.877
2	wiped	12.9	12.0	9.8	6.9	5.1	3.5	2.1	1.6	0.992	53.9	0.970
3	unwiped	16.8	15.7	12.0	9.2	5.3	3.7	2.5	1.9	0.840	67.1	0.857
3	unwiped	23.2	17.3	14.2	12.8	10.7	6.7	3.7	2.4	1.160	91.0	1.162
3	unwiped	19.3	12.5	10.9	9.6	7.9	6.4	4.3	2.9	0.965	73.8	0.942
3	unwiped	20.8	15.3	12.0	10.7	8.2	5.3	3.2	1.9	1.040	77.4	0.988
3	unwiped	19.9	15.2	11.4	10.7	9.5	7.2	5.1	3.3	0.995	82.3	1.051
4	wiped	6.9	8.6	8.0	7.2	5.7	4.7	3.7	2.8	0.898	47.6	1.099
4	wiped	7.6	8.2	7.5	6.6	5.2	4.2	3.1	2.5	0.990	44.9	1.028
4	wiped	8.0	7.3	6.4	5.0	4.0	3.1	2.5	2.2	1.042	38.5	0.881
4	wiped	8.0	7.9	7.4	6.2	4.9	4.0	3.1	2.5	1.042	44.0	1.007
4	wiped	7.9	8.1	7.2	6.3	4.8	3.7	2.9	2.5	1.029	43.8	0.994
5	unwiped	9.4	9.2	7.4	5.5	3.8	2.9	2.1	1.6	1.011	41.9	0.890
5	unwiped	9.0	8.3	8.9	8.1	6.0	3.7	2.4	1.7	0.968	48.1	1.022
5	unwiped	7.3	8.3	8.0	6.3	4.5	3.1	2.4	1.5	0.785	41.4	0.879
5	unwiped	13.4	7.8	8.2	8.6	8.0	6.3	4.5	3.0	1.441	59.8	1.270
5	unwiped	7.4	7.5	8.3	6.8	5.4	3.9	2.9	2.0	0.796	44.2	0.939
6	wiped	8.0	7.1	5.8	4.5	3.2	2.6	1.6	1.0	0.976	33.8	0.863
6	wiped	7.8	8.4	7.7	6.0	4.7	3.7	2.7	1.8	0.951	42.8	1.093
6	wiped	8.3	8.0	7.3	5.2	4.2	3.3	2.4	1.4	1.012	40.1	1.024
6	wiped	8.4	7.9	6.7	5.2	3.8	3.2	2.3	1.3	1.024	38.8	0.991
6	wiped	8.5	8.1	6.9	5.3	4.3	3.3	2.4	1.5	1.037	40.3	1.029

Key to table (see general key at beginning of Appendix 3)

0µm/mean : Pixel count at 0µm expressed as a ratio to the mean of the pixel counts at 0µm for that well

tot p/mean : Total pixel count expressed as a ratio to the mean of the total pixel counts for that well

Table 7: Median and 95% Confidence intervals for the first assay of MDA-MB-231 / EGF stimulated invasion of the MDA-MB-231 cell line

EGF	0µm	3µm	6µm	9µm	12µm	15µm	18µm	21µm	tot p	s tot p	ratio	s ratio
0	18.5	16.2	8.7	3.1	1.0	0.5	0.3	0.1	48.4	0.851	0.021	0.153
0	15.3	8.1	2.4	0.9	0.5	0.2	0.1	0.0	27.5	0.283	0.012	0.068
0	14.1	7.2	2.0	0.5	0.2	0.1	0.0	0.0	24.1	0.190	0.004	0.000
0	10.2	5.4	1.6	0.5	0.2	0.1	0.0	0.0	18.0	0.024	0.006	0.014
0	15.7	12.0	6.0	2.1	0.8	0.3	0.1	0.1	37.1	0.543	0.015	0.098
1	19.6	13.4	6.4	2.6	1.2	0.5	0.3	0.1	44.1	0.734	0.023	0.173
1	13.3	7.4	2.7	1.1	0.6	0.3	0.1	0.1	25.6	0.231	0.021	0.159
1	15.3	10.6	4.6	1.7	0.6	0.3	0.1	0.1	33.3	0.442	0.016	0.113
1	20.1	13.6	7.3	2.9	1.3	0.5	0.2	0.1	46.0	0.785	0.020	0.142
1	19.8	9.2	4.4	0.9	0.7	0.3	0.1	0.1	35.5	0.500	0.015	0.099
5	9.0	8.2	4.1	2.1	1.0	0.6	0.3	0.2	25.5	0.228	0.052	0.441
5	9.5	6.5	3.0	1.2	0.7	0.3	0.1	0.1	21.4	0.117	0.026	0.205
5	8.4	7.0	4.0	1.6	0.7	0.5	0.3	0.1	22.6	0.149	0.046	0.392
5	9.1	7.6	5.6	3.4	1.3	0.6	0.3	0.2	28.1	0.299	0.049	0.419
5	12.7	6.9	2.7	1.3	0.6	0.3	0.1	0.1	24.7	0.207	0.022	0.169
10	9.7	6.2	3.2	1.6	1.0	0.6	0.2	0.1	22.6	0.149	0.047	0.399
10	11.6	10.8	8.8	5.5	2.6	1.1	0.5	0.4	41.3	0.658	0.064	0.557
10	12.0	6.3	4.5	2.5	1.3	0.8	0.5	0.3	28.2	0.302	0.070	0.613
10	7.9	5.1	2.5	1.0	0.5	0.3	0.1	0.1	17.5	0.011	0.032	0.260
10	7.8	12.5	13.7	10.4	5.7	2.4	1.0	0.4	53.9	1.000	0.112	1.000
30	14.3	13.4	8.9	4.4	2.0	1.0	0.6	0.3	44.9	0.755	0.052	0.443
30	6.6	5.5	2.5	1.3	0.6	0.4	0.1	0.1	17.1	0.000	0.041	0.342
30	9.4	7.9	4.9	2.3	0.9	0.4	0.2	0.2	26.2	0.247	0.036	0.295
30	7.6	7.5	4.0	1.9	1.0	0.4	0.2	0.1	22.7	0.152	0.037	0.301
30	11.9	13.5	9.1	3.8	1.5	0.6	0.3	0.1	40.8	0.644	0.029	0.230

Key to table (see general key at beginning of Appendix 3)

Table 7 (cont.): Median and 95% Confidence intervals for the second assay of MDA-MB-231 / EGF stimulated invasion of the MDA-MB-231 cell line

EGF	0µm	3µm	6µm	9µm	12µm	15µm	18µm	21µm	tot p	s tot p	ratio	s ratio
0	10.2	9.7	10.0	7.4	3.4	1.1	0.3	0.1	42.2	0.269	0.050	0.064
0	13.2	11.8	8.8	4.8	2.3	1.0	0.5	0.2	42.6	0.273	0.050	0.064
0	9.2	9.5	8.9	4.8	1.8	0.5	0.2	0.1	35.0	0.213	0.029	0.000
0	9.0	8.2	6.1	2.8	0.9	0.4	0.2	0.1	27.7	0.156	0.030	0.003
0	6.9	7.2	4.7	2.2	0.8	0.3	0.2	0.1	22.4	0.114	0.032	0.009
1	6.6	7.3	6.5	5.2	3.2	1.7	0.8	0.3	31.6	0.186	0.137	0.325
1	7.4	9.6	8.3	5.5	2.9	1.2	0.4	0.2	35.5	0.217	0.071	0.127
1	7.9	5.0	3.9	2.9	1.6	0.8	0.4	0.2	22.7	0.117	0.083	0.163
1	8.6	8.0	6.6	4.8	2.4	1.2	0.4	0.2	32.2	0.191	0.078	0.146
1	7.2	5.8	4.6	2.6	2.1	0.4	0.2	0.1	23.0	0.119	0.040	0.032
5	11.3	12.8	9.3	5.4	3.5	2.4	2.1	1.8	48.6	0.319	0.189	0.480
5	12.2	12.0	9.1	5.7	3.3	2.1	1.3	1.0	46.7	0.305	0.132	0.310
5	3.9	3.7	2.9	2.1	1.3	0.7	0.4	0.2	15.2	0.058	0.124	0.285
5	5.7	4.3	3.4	1.9	1.1	0.6	0.4	0.3	17.7	0.078	0.097	0.204
5	8.7	9.4	6.0	3.8	2.4	1.2	0.6	0.4	32.5	0.193	0.091	0.187
10	13.6	17.9	14.1	10.9	9.2	6.9	5.6	4.0	82.2	0.583	0.362	1.000
10	22.5	24.5	20.3	15.5	10.7	6.9	4.5	3.0	107.9	0.784	0.214	0.556
10	14.2	15.2	12.6	8.2	5.4	2.8	2.2	1.4	62.0	0.424	0.152	0.371
10	27.2	23.7	20.7	15.4	9.9	7.3	5.2	3.1	112.5	0.820	0.218	0.567
10	25.8	27.2	23.7	19.1	13.6	11.6	8.6	5.9	135.5	1.000	0.340	0.935
30	25.0	19.8	15.1	8.1	5.6	2.7	2.2	0.9	79.4	0.561	0.097	0.204
30	16.6	19.9	15.7	12.3	7.1	4.0	2.6	1.8	80.0	0.565	0.161	0.396
30	9.9	10.2	6.2	1.0	2.1	1.0	1.0	0.8	32.2	0.191	0.106	0.233
30	13.3	12.8	7.8	5.1	2.5	1.6	0.8	0.4	44.3	0.286	0.083	0.161
30	16.3	10.4	9.4	4.3	3.4	1.8	1.0	0.6	47.2	0.309	0.094	0.196
50	11.8	9.9	6.8	4.0	2.4	1.4	0.9	0.6	37.8	0.235	0.102	0.219
50	5.1	2.1	1.2	0.8	0.4	0.3	0.2	0.1	10.2	0.019	0.071	0.128
50	7.8	7.7	5.7	3.9	2.0	1.3	1.0	0.7	30.1	0.175	0.142	0.338
50	2.2	2.3	1.5	0.7	0.5	0.3	0.2	0.1	7.8	0.000	0.100	0.213
50	1.8	2.9	2.0	0.9	0.5	0.3	0.3	0.2	8.9	0.009	0.119	0.272

Key to table (see general key at beginning of Appendix 3)

Table 7 (cont.): Median and 95% Confidence intervals for the third assay of MDA-MB-231 / EGF stimulated invasion of the MDA-MB-231 cell line

EGF	0μm	3μm	6μm	9μm	12μm	15μm	18μm	21μm	tot p	s tot p	ratio	s ratio
0	11.7	14.5	11.3	6.2	2.4	0.7	0.4	0.2	47.4	0.245	0.035	0.017
0	11.3	14.0	7.7	3.6	1.2	0.6	0.4	0.1	38.9	0.189	0.033	0.013
0	12.0	14.5	13.0	9.2	4.4	1.4	0.5	0.2	55.2	0.296	0.053	0.084
0	15.0	19.5	11.2	6.0	3.0	1.4	0.7	0.2	57.0	0.308	0.050	0.074
0	8.5	10.8	5.9	2.9	1.2	0.4	0.3	0.1	30.0	0.130	0.030	0.000
1	10.6	13.2	8.2	5.8	2.8	1.6	0.5	0.3	43.0	0.216	0.075	0.162
1	8.8	11.3	5.7	3.2	2.5	0.6	0.3	0.1	32.5	0.147	0.039	0.032
1	8.3	10.2	8.0	6.5	4.0	2.2	1.0	0.5	40.7	0.201	0.140	0.396
1	9.6	12.2	5.1	3.2	1.8	1.2	0.6	0.3	34.0	0.157	0.078	0.173
1	9.2	11.5	10.3	6.9	3.6	1.5	0.5	0.3	43.8	0.221	0.074	0.159
5	23.5	25.6	15.3	10.6	5.6	4.3	3.0	2.1	90.0	0.526	0.146	0.417
5	8.5	8.7	5.8	4.1	2.9	2.3	1.8	1.5	35.6	0.167	0.243	0.768
5	20.0	17.9	13.9	9.0	5.6	4.1	2.9	2.1	75.5	0.430	0.176	0.524
5	21.6	23.2	15.2	9.2	6.0	4.5	3.3	2.9	85.9	0.499	0.178	0.534
5	16.3	17.6	9.8	6.1	3.8	2.4	1.5	0.8	58.3	0.317	0.108	0.279
10	16.8	21.2	17.5	12.9	10.3	7.8	5.3	4.0	95.8	0.564	0.308	1.000
10	27.3	34.2	22.0	17.1	11.2	7.0	4.2	3.1	126.1	0.764	0.171	0.508
10	32.0	40.6	23.4	18.6	12.0	8.9	5.4	3.5	144.4	0.884	0.185	0.559
10	30.5	38.5	28.2	22.9	15.6	11.9	8.4	6.0	162.0	1.000	0.271	0.865
10	16.8	20.9	15.6	9.9	6.5	3.3	2.6	1.4	77.0	0.440	0.137	0.385
30	16.6	19.9	9.7	6.3	3.1	2.0	1.1	0.5	59.2	0.323	0.078	0.173
30	28.1	35.2	16.2	10.1	7.0	3.2	2.6	1.0	103.4	0.614	0.086	0.200
30	19.8	23.4	17.2	12.8	7.3	4.9	3.0	2.1	90.5	0.529	0.166	0.488
30	20.3	25.5	11.6	5.5	4.2	2.2	1.3	0.8	71.4	0.403	0.075	0.162
30	11.6	14.5	7.8	1.3	2.4	1.3	1.3	0.9	41.1	0.204	0.103	0.264
50	12.9	15.6	8.4	4.8	3.0	1.7	1.1	0.8	48.3	0.251	0.098	0.243
50	6.3	7.9	1.5	1.0	0.5	0.3	0.3	0.1	17.9	0.051	0.045	0.053
50	9.8	12.0	7.1	4.9	2.4	1.6	1.2	0.8	39.8	0.195	0.125	0.340
50	2.3	2.8	2.4	1.1	0.6	0.4	0.4	0.3	10.3	0.001	0.147	0.420
50	2.7	3.3	1.9	0.9	0.6	0.4	0.3	0.1	10.2	0.000	0.101	0.257

Key to table (see general key at beginning of Appendix 3)

Table 8: EGF stimulated invasion of the BT20 cell line (Assay 1)

EGF	0µm	3µm	6µm	9µm	12µm	15µm	tot p	s tot p	ratio	s ratio
0	1.1	1.1	0.7	0.5	0.2	0.2	3.8	0.173	0.310	0.418
0	0.7	0.7	0.4	0.2	0.2	0.1	2.3	0.080	0.278	0.333
0	0.7	0.8	0.4	0.4	0.2	0.2	2.7	0.105	0.421	0.707
0	2.4	1.0	0.6	0.3	0.2	0.1	4.6	0.222	0.150	0.000
0	1.2	1.0	0.5	0.3	0.2	0.2	3.4	0.148	0.259	0.285
1	4.1	4.7	3.6	2.2	1.7	0.9	17.2	1.000	0.387	0.619
1	3.0	2.5	1.6	1.2	0.8	0.6	9.7	0.537	0.366	0.564
1	4.2	2.8	1.8	0.9	0.5	0.6	10.8	0.605	0.227	0.202
1	5.2	4.3	2.9	1.6	1.0	0.9	15.9	0.920	0.282	0.345
1	3.8	3.9	2.6	1.8	1.1	0.9	14.1	0.809	0.369	0.571
5	3.8	4.0	3.7	2.8	1.8	0.8	16.9	0.981	0.470	0.834
5	3.3	3.5	2.8	1.4	0.8	0.8	12.6	0.716	0.313	0.424
5	4.5	3.2	1.7	0.9	0.6	0.5	11.4	0.642	0.213	0.164
5	4.9	3.7	2.6	1.3	0.8	0.6	13.9	0.796	0.241	0.238
5	3.7	3.3	3.1	2.1	1.4	0.9	14.5	0.833	0.436	0.745
10	2.5	2.0	1.7	1.1	0.7	0.6	8.6	0.469	0.387	0.619
10	3.8	2.4	1.5	1.0	0.6	0.3	9.6	0.531	0.247	0.252
10	4.3	3.6	2.2	1.4	0.5	0.4	12.4	0.704	0.228	0.203
10	2.8	2.8	1.7	0.9	0.6	0.4	9.2	0.506	0.260	0.288
10	2.3	2.0	1.2	0.6	0.4	0.3	6.8	0.358	0.236	0.225
30	7.3	2.5	1.9	0.8	0.8	0.7	14.0	0.802	0.197	0.122
30	2.6	2.5	2.2	1.3	1.0	0.6	10.2	0.568	0.397	0.645
30	5.4	1.7	0.9	0.9	0.6	0.3	9.8	0.543	0.225	0.196
30	0.9	0.9	0.9	0.6	0.4	0.3	4.0	0.185	0.481	0.865
30	0.9	0.9	0.7	0.4	0.3	0.3	3.5	0.154	0.400	0.652
50	0.6	0.5	0.4	0.4	0.2	0.2	2.3	0.080	0.533	1.000
50	0.5	0.4	0.3	0.2	0.1	0.1	1.6	0.037	0.333	0.478
50	0.3	0.3	0.2	0.1	0.1	0.0	1.0	0.000	0.250	0.261

Key to table (see general key at beginning of Appendix 3)

Table 8 (cont.): EGF stimulated invasion of the BT20 cell line (Assay 2)

EGF	0µm	3µm	6µm	9µm	12µm	15µm	tot p	s tot p	ratio	s ratio
0	0.4	0.3	0.3	0.2	0.2	0.2	1.6	0.011	0.600	1.000
0	1.0	1.0	0.8	0.5	0.4	0.3	4.0	0.096	0.429	0.503
0	0.4	0.3	0.2	0.2	0.2	0.1	1.4	0.004	0.556	0.874
1	6.4	5.2	4.2	3.5	2.6	1.9	23.8	0.804	0.506	0.730
1	6.3	5.6	4.4	3.2	2.5	1.8	23.8	0.804	0.460	0.596
1	3.0	2.4	1.5	0.9	0.6	0.4	8.8	0.268	0.275	0.062
5	7.8	6.9	5.0	4.1	3.0	2.5	29.3	1.000	0.487	0.674
5	5.7	5.1	3.9	2.9	2.4	1.7	21.7	0.729	0.476	0.642
5	2.9	2.2	1.2	0.8	0.5	0.3	7.9	0.236	0.254	0.000
10	5.9	3.8	2.5	1.8	0.9	0.6	15.5	0.507	0.270	0.048
10	2.1	1.8	1.3	0.9	0.7	0.6	7.4	0.218	0.423	0.489
10	2.0	1.7	1.0	0.8	0.5	0.3	6.3	0.179	0.340	0.250
30	1.2	0.8	0.5	0.4	0.3	0.2	3.4	0.075	0.360	0.306
30	1.3	0.6	0.5	0.4	0.3	0.2	3.3	0.071	0.375	0.350
30	1.3	0.9	0.6	0.5	0.4	0.2	3.9	0.093	0.393	0.401
50	0.8	0.6	0.5	0.3	0.3	0.2	2.7	0.050	0.421	0.483
50	0.5	0.3	0.2	0.2	0.1	0.0	1.3	0.000	0.300	0.133
50	0.6	0.4	0.3	0.2	0.2	0.1	1.8	0.018	0.385	0.378

Key to table (see general key at beginning of Appendix 3)

Table 9: EGF stimulated invasion of the MCF7ADR cell line (Assay 1)

EGF	0µm	3µm	6µm	9µm	12µm	15µm	18µm	21µm	tot p	s tot p	ratio	s ratio
0	13.3	9.1	5.1	2.2	0.7	0.2	0.1	0.0	30.7	0.458	0.011	0.034
0	14.3	11.1	5.8	3.0	2.1	0.8	0.5	0.2	37.8	0.650	0.048	0.401
0	10.5	12.7	10.3	7.3	2.9	1.6	0.7	0.2	46.2	0.876	0.075	0.664
1	22.0	11.3	7.6	3.9	2.5	1.9	1.0	0.6	50.8	1.000	0.086	0.772
1	16.5	12.7	7.1	3.0	1.9	1.1	0.8	0.4	43.5	0.803	0.063	0.552
1	12.3	9.1	5.3	3.5	3.0	1.6	0.8	0.5	36.1	0.604	0.109	1.000
5	8.3	9.6	5.0	2.7	0.9	0.3	0.1	0.0	26.9	0.356	0.017	0.098
5	9.9	8.6	5.4	3.3	1.6	1.0	0.6	0.2	30.6	0.456	0.075	0.671
5	5.2	4.5	3.0	1.9	1.1	0.5	0.2	0.1	16.5	0.075	0.063	0.549
10	9.3	7.3	4.8	1.5	0.8	0.3	0.1	0.0	24.1	0.280	0.019	0.111
10	6.5	5.9	2.5	1.0	0.6	0.6	0.4	0.1	17.6	0.105	0.074	0.656
10	6.3	5.2	3.7	1.5	1.1	0.5	0.3	0.1	18.7	0.135	0.059	0.513
30	5.7	5.4	4.1	2.4	0.9	0.3	0.2	0.0	19.0	0.143	0.033	0.256
30	5.8	4.7	2.8	1.3	0.3	0.1	0.0	0.0	15.0	0.035	0.008	0.000
30	4.6	4.0	2.9	1.5	0.6	0.1	0.0	0.0	13.7	0.000	0.009	0.011

Key to table (see general key at beginning of Appendix 3)

Table 9 (cont.): EGF stimulated invasion of the MCF7ADR cell line (Assay 2)

EGF	0μm	3μm	6μm	9μm	12μm	15μm	18μm	21μm	tot p	s tot p	ratio	s ratio
0	11.1	8.6	4.9	2.1	0.9	0.4	0.3	0.1	28.4	0.492	0.033	0.127
0	7.4	8.1	5.1	2.3	1.6	0.8	0.6	0.2	26.1	0.430	0.078	0.345
0	15.0	12.2	9.7	5.7	2.3	1.2	0.6	0.4	47.1	1.000	0.060	0.258
1	20.2	6.7	4.9	2.3	1.6	0.9	0.6	0.6	37.8	0.748	0.066	0.289
1	5.4	4.7	4.0	2.2	1.2	0.7	0.7	0.5	19.4	0.247	0.135	0.620
1	4.1	3.0	3.2	3.3	2.1	1.1	0.6	0.5	17.9	0.207	0.214	1.000
5	6.2	6.9	3.8	2.0	0.7	0.2	0.1	0.0	19.9	0.261	0.018	0.056
5	5.1	4.3	2.7	1.3	0.5	0.2	0.2	0.1	14.4	0.111	0.041	0.170
5	3.5	3.2	2.2	0.9	0.3	0.1	0.1	0.0	10.3	0.000	0.022	0.079
10	7.2	5.8	3.4	1.1	0.1	0.1	0.0	0.0	17.7	0.201	0.006	0.000
10	4.6	4.2	1.8	0.6	0.4	0.4	0.2	0.1	12.3	0.054	0.066	0.289
10	4.6	4.4	2.8	1.2	0.7	0.4	0.2	0.1	14.4	0.111	0.059	0.257
30	4.2	4.0	3.3	2.0	0.8	0.2	0.1	0.0	14.6	0.117	0.026	0.097
30	4.1	3.5	2.3	1.0	0.3	0.1	0.0	0.0	11.3	0.027	0.010	0.019
30	3.4	3.0	2.4	1.2	0.5	0.1	0.0	0.0	10.6	0.008	0.011	0.025

Key to table (see general key at beginning of Appendix 3)

Table 10: Adding ICR16 above the Matrigel layer

ICR16	0µm	3µm	6µm	9µm	12µm	15µm	18µm	21µm	tot p	ratio	s ratio
0	16.8	13.2	7.7	3.7	2.4	1.6	0.8	0.3	46.5	0.072	0.387
0	13.5	10.7	5.6	2.4	1.4	0.9	0.4	0.2	35.1	0.050	0.224
0	10.1	8.8	6.3	3.6	2.2	1.1	0.8	0.4	33.3	0.091	0.538
2	12.1	10.0	7.7	4.9	3.1	2.2	1.4	0.9	42.3	0.151	0.998
2	9.8	8.4	5.8	3.3	1.6	0.7	0.6	0.4	30.6	0.071	0.381
2	7.8	7.2	5.1	3.0	1.7	1.3	0.7	0.5	27.3	0.124	0.793
10	13.3	12.2	9.6	4.9	3.0	1.3	0.6	0.3	45.2	0.063	0.319
10	10.6	7.7	4.0	2.5	1.7	1.3	0.9	0.3	29.0	0.112	0.699
10	8.3	7.5	5.0	3.0	1.5	0.9	0.7	0.5	27.4	0.101	0.613
100	9.8	6.7	3.3	1.7	1.1	0.7	0.3	0.2	23.8	0.061	0.303
100	8.6	7.3	4.1	2.1	1.4	0.9	0.6	0.4	25.4	0.095	0.567
100	7.8	4.7	2.8	1.4	0.8	0.5	0.3	0.1	18.4	0.059	0.289
500	11.9	10.9	7.9	4.6	2.5	1.7	1.1	0.7	41.3	0.114	0.713
500	5.0	4.0	2.9	2.2	1.6	1.0	0.5	0.3	17.5	0.151	1.000
500	8.3	7.9	5.3	3.1	1.9	1.2	0.8	0.4	28.9	0.112	0.695
5000	3.0	1.1	0.6	0.4	0.2	0.1	0.0	0.0	5.4	0.021	0.000
5000	2.5	1.4	0.7	0.3	0.1	0.1	0.0	0.0	5.1	0.022	0.004
5000	2.7	1.8	0.9	0.4	0.2	0.1	0.1	0.0	6.2	0.037	0.121

Key to table (see general key at beginning of Appendix 3)

ICR16 : Nanograms ICR16 antibody added above the Matrigel layer

Table 11: Adding antibody below the Matrigel layer / Plot of the median standardised pixel counts for the ICR antibodies

ICR9	0µm	3µm	6µm	9µm	12µm	15µm	18µm	21µm	tot p	s tot p	ratio	s ratio
0	15.1	13.9	10.2	6.5	4.3	3.1	2.0	1.2	56.3	0.949	0.161	0.660
0	14.5	13.1	9.6	6.2	4.1	2.8	1.7	1.0	53.0	0.882	0.148	0.590
0	15.8	14.6	9.5	5.1	2.8	1.7	0.9	0.5	50.9	0.841	0.078	0.204
10	13.4	12.9	9.4	6.0	3.8	2.7	1.7	1.0	50.9	0.841	0.151	0.609
10	11.4	10.3	7.2	4.4	2.7	1.7	1.0	0.7	39.4	0.612	0.118	0.424
10	10.9	10.0	7.1	4.3	2.5	1.5	0.9	0.5	37.7	0.578	0.104	0.347
50	12.5	11.5	7.9	4.5	2.7	1.8	1.1	0.6	42.6	0.675	0.110	0.380
50	15.9	15.6	10.8	6.7	4.2	3.0	1.8	0.9	58.9	1.000	0.135	0.518
50	13.4	13.0	9.0	5.2	3.3	2.2	1.3	0.7	48.1	0.785	0.119	0.430
100	9.7	10.0	7.7	4.8	2.9	1.8	1.2	0.7	38.8	0.600	0.135	0.520
100	10.3	8.1	5.0	3.0	1.9	1.2	0.7	0.4	30.6	0.436	0.098	0.317
100	9.3	8.4	5.8	3.3	2.0	1.2	0.7	0.3	31.0	0.444	0.097	0.292
400	5.9	6.3	5.8	4.4	2.9	1.9	1.3	0.8	29.3	0.410	0.222	1.000
400	3.3	3.3	2.6	1.7	1.1	0.7	0.4	0.2	13.3	0.092	0.141	0.554
400	3.3	3.3	2.6	1.7	0.9	0.5	0.3	0.1	12.7	0.080	0.098	0.315
1000	3.4	2.6	1.7	1.0	0.6	0.4	0.2	0.1	10.0	0.026	0.091	0.277
1000	5.0	4.5	2.8	1.3	0.6	0.3	0.1	0.1	14.7	0.120	0.041	0.000
1000	3.2	2.4	1.3	0.7	0.5	0.3	0.2	0.1	8.7	0.000	0.087	0.255

Key to table (see general key at beginning of Appendix 3)

ICR9 : Nanograms ICR9 antibody added below the Matrigel layer

Table 12: Adding antibody below the Matrigel layer / Plot of the median standardised pixel counts for the ICR antibodies

Antibody	ICR16 Assay 1	ICR16 Assay 2	s Assay 1	s Assay 2	s ICR9l
0	22.0	24.20	1.000	1.000	0.937
0	16.6	12.80	0.749	0.521	0.890
0	12.5	17.30	0.558	0.710	0.992
0	20.7	19.40	0.940	0.798	
0	15.1	18.20	0.679	0.748	
10	4.2	6.50	0.172	0.256	0.803
10	3.7	4.10	0.149	0.155	0.646
10	2.9	4.60	0.112	0.176	0.606
10	1.8	1.98	0.060	0.066	
10	2.1	2.20	0.074	0.076	
50	2.6	3.70	0.098	0.139	0.732
50	2.2	3.10	0.079	0.113	1.000
50	2.9	3.19	0.112	0.117	0.803
50	1.9	1.60	0.065	0.050	
50	1.3	1.43	0.037	0.043	
100	1.5	2.00	0.047	0.067	0.512
100	1.3	1.30	0.037	0.038	0.559
100	1.2	1.50	0.033	0.046	0.480
100	1.3	1.43	0.037	0.043	
100	1.4	1.40	0.042	0.042	
400	2.3	2.60	0.084	0.092	0.213
400	1.7	2.20	0.056	0.076	0.008
400	0.5	0.80	0.000	0.017	0.008
400	1.2	1.32	0.033	0.039	
400	1.4	1.70	0.042	0.055	
1000	1.1	1.40	0.028	0.042	0.016
1000	1.1	1.21	0.028	0.034	0.142
1000	1.2	0.80	0.033	0.017	0.000
1000	0.7	0.40	0.009	0.000	
1000	0.5	0.70	0.000	0.013	

Key to table (see general key at beginning of Appendix 3)
Antibody : nanograms ICR9 / ICR16 added below the filter

Table 13: Determining the significance of DMSO on MDA-MB-231 cell proliferation

DMSO (%)	Counts					mean	Std Dev.	LM
0	16	15	13	17	11	14.4	2.4	normal cell appearance, confluent
0.1	16	20	18	15	24	18.6	3.6	normal cell appearance, confluent
0.25	13	8	15	14	20	14.0	4.3	normal cell appearance, confluent
0.5	17	17	9	10	14	13.4	3.8	normal cell appearance, confluent
1	16	19	15	10	20	16.0	3.9	normal cell appearance, confluent
2	14	14	12	12	13	13.0	1.0	cells appear less healthy and are sub-confluent

Key to table (see general key at beginning of Appendix 3)

DMSO (%) : DMSO in Culture medium (volume per volume)

Counts : Cells per unit volume measured with a haemocytometer

LM : Light microscopy appearance of the cells prior to trypsinisation for counting

Table 14 : Using tyrosine kinase inhibitor in the invasion assay

assay	TKI	0μm	3μm	6μm	9μm	12μm	15μm	18μm	21μm	tot p	s tot p	ratio	s ratio
1	0	6.2	4.3	2.5	1.6	1.1	0.8	0.7	0.6	17.8	0.099	0.162	0.377
1	0	14.2	13.1	12.0	10.3	8.3	6.0	4.2	2.7	70.8	1.000	0.328	0.853
1	0	4.5	3.5	2.2	1.1	0.4	0.2	0.1	0.0	12.0	0.000	0.029	0.000
1	0	12.7	10.6	8.9	6.9	5.2	3.5	2.4	1.7	51.9	0.679	0.236	0.590
1	1	14.2	12.8	10.4	7.9	5.7	3.9	2.7	1.8	59.4	0.806	0.225	0.557
1	1	13.0	12.9	11.5	9.2	7.2	5.8	4.7	3.7	68.0	0.952	0.380	1.000
1	1	8.0	8.0	6.6	4.5	2.8	1.7	1.0	0.6	33.2	0.361	0.146	0.333
1	5	7.3	7.7	7.0	5.5	3.7	2.3	1.3	0.7	35.5	0.400	0.195	0.474
1	5	7.9	7.9	6.5	4.4	2.7	1.4	0.7	0.4	31.9	0.338	0.112	0.236
1	5	7.0	6.6	5.7	3.5	2.2	1.5	1.0	0.5	28.0	0.272	0.155	0.360
1	5	14.3	13.3	11.2	8.7	6.8	5.1	4.1	3.3	66.8	0.932	0.322	0.836
1	10	4.1	4.2	3.7	2.7	1.7	0.9	0.4	0.2	17.9	0.100	0.125	0.273
1	10	5.4	5.2	4.3	2.9	1.6	0.8	0.3	0.1	20.6	0.146	0.081	0.146
1	10	2.8	3.1	3.0	2.5	1.6	0.8	0.3	0.1	14.2	0.037	0.135	0.301
2	0	12.9	12.7	11.7	10.5	8.7	6.7	4.6	2.9	70.7	1.000	0.381	0.935
2	0	10.4	9.1	7.6	6.2	4.6	3.1	1.8	1.0	43.8	0.559	0.218	0.504
2	0	13.6	12.9	10.5	8.0	3.9	1.8	0.7	0.2	51.6	0.687	0.073	0.120
2	1	10.7	9.8	8.0	6.0	4.2	2.9	1.9	1.2	44.7	0.574	0.211	0.485
2	1	11.5	11.1	9.7	7.8	5.7	4.5	3.5	2.7	56.5	0.767	0.331	0.804
2	1	12.5	11.9	10.4	8.5	6.9	5.6	4.7	3.8	64.3	0.895	0.405	1.000
2	5	6.3	6.5	5.4	4.0	2.7	1.7	1.0	0.5	28.1	0.302	0.176	0.393
2	5	8.8	8.0	6.1	4.4	2.8	1.7	0.9	0.5	33.2	0.385	0.135	0.286
2	5	9.4	9.1	7.1	4.7	2.7	1.4	0.9	0.5	35.8	0.428	0.109	0.217
2	10	2.4	2.4	2.1	1.5	0.9	0.3	0.1	0.0	9.7	0.000	0.058	0.081
2	10	5.7	6.0	5.2	3.7	2.1	1.0	0.3	0.1	24.1	0.236	0.083	0.147
2	10	6.7	6.5	5.0	3.1	1.4	0.4	0.1	0.0	23.2	0.221	0.027	0.000

Key to table (see general key at beginning of Appendix 3)

TKI : Concentration of tyrosine kinase inhibitor in μM

Table 15: Invasion and proliferation in the tyrosine kinase inhibitor experiments

TKI	bw p	tot p	s tot p	s bw p	sum p	s sum p
0	16.5	70.7	1.000	0.459	87.2	1.000
0	11.3	43.8	0.620	0.314	55.1	0.631
0	5.8	51.6	0.730	0.160	57.4	0.658
5	22.4	28.1	0.397	0.625	50.5	0.579
5	32.1	33.2	0.470	0.896	65.3	0.749
5	20.4	35.8	0.506	0.569	56.2	0.644
10	15.6	9.7	0.137	0.434	25.3	0.289
10	14.1	24.1	0.341	0.392	38.2	0.437
10	20.0	23.2	0.328	0.557	43.2	0.495
50	8.5	0.0	0.000	0.235	8.5	0.096
50	11.6	0.0	0.000	0.322	11.6	0.132
50	12.0	0.0	0.000	0.333	12.0	0.137
100	0.1	0.0	0.000	0.000	0.1	0.000
100	0.2	0.0	0.000	0.003	0.2	0.001
100	0.1	0.0	0.000	0.000	0.1	0.000

Key to table (see general key at beginning of Appendix 3)

TKI : concentration of tyrosine kinase inhibitor in μM
tot p: sum of fluorescing pixels from 0-21μm
bw p : fluorescing pixels below the filter (before wiping the filter)
sum p: in this assay 'tot p' refers to the sum of pixels from tot p' and 'bw p'

Table 16: Invasion induced by TGF-β

TGF-β	0μm	3μm	6μm	9μm	12μm	15μm	18μm	21μm	tot p	ratio	s ratio
0	19.8	20.0	15.8	9.4	5.9	3.8	2.7	1.4	78.8	0.142	0.276
0	11.3	8.8	5.8	3.4	3.0	2.2	1.4	0.6	36.5	0.162	0.344
0	17.6	15.9	9.6	5.2	3.2	1.3	0.9	0.4	54.1	0.060	0.000
0.5	12.9	12.3	9.0	5.9	4.8	3.6	2.3	1.3	52.1	0.211	0.508
0.5	10.0	9.4	5.7	3.4	3.0	2.0	1.4	0.6	35.5	0.159	0.335
0.5	11.2	12.4	10.5	6.1	4.2	2.7	2.0	1.3	50.4	0.176	0.391
2.5	15.4	13.8	9.6	4.3	5.8	4.5	2.6	1.4	57.4	0.219	0.537
2.5	16.0	10.9	8.5	5.5	3.2	2.5	1.4	0.9	48.9	0.136	0.255
2.5	8.5	5.3	4.7	4.3	3.8	3.0	1.8	1.0	32.4	0.314	0.856
5	8.8	8.9	8.7	6.5	5.3	3.6	3.1	2.7	47.6	0.356	1.000
5	13.8	12.3	10.0	7.8	6.0	4.7	3.5	2.2	60.3	0.288	0.770
5	9.0	9.3	6.2	4.7	3.2	3.6	2.1	1.6	39.7	0.298	0.804
10	15.5	15.3	10.9	6.6	4.8	3.6	2.1	1.6	60.4	0.175	0.388
10	10.5	12.6	9.4	5.8	4.0	3.1	2.6	1.8	49.8	0.231	0.576
10	11.2	10.2	7.8	5.4	3.4	3.0	2.8	1.6	45.4	0.253	0.653
30	7.4	8.1	7.4	4.3	2.7	1.9	1.6	0.9	34.3	0.192	0.446
30	7.8	7.4	5.7	3.7	1.6	1.9	1.4	1.0	30.5	0.206	0.492
30	10.2	10.1	6.6	5.1	2.5	1.9	1.5	0.6	38.5	0.149	0.299

Key to table (see general key at beginning of Appendix 3)

TGF-β : nanograms TGF-β added above the Matrigel layer

Table 16 (cont.): Invasion induced by TGF-β

ng TGF-β	0μm	3μm	6μm	9μm	12μm	15μm	18μm	21μm	tot p	ratio	s ratio
0	14.2	9.2	5.7	3.9	3.2	1.8	1.1	0.5	39.6	0.117	0.191
0	5.4	5.3	3.6	1.8	1.1	0.9	0.6	0.3	19.0	0.126	0.222
0	15.7	13.9	9.6	4.3	3.1	2.0	1.0	0.5	50.1	0.089	0.098
0.5	15.6	12.6	8.8	5.9	4.7	3.4	1.7	0.9	53.6	0.162	0.344
0.5	3.9	3.5	2.1	1.2	0.8	0.7	0.3	0.3	12.8	0.137	0.259
0.5	8.5	6.8	4.5	2.9	2.2	1.4	1.0	0.4	27.7	0.141	0.274
2.5	9.0	10.9	8.3	6.0	4.2	3.1	2.3	1.6	45.4	0.248	0.635
2.5	10.5	10.5	8.4	5.5	4.7	2.6	1.8	1.0	45.0	0.184	0.417
2.5	12.0	9.9	9.0	4.8	3.5	3.1	1.9	1.0	45.2	0.194	0.453
5	9.4	9.4	7.9	5.2	4.4	3.3	2.1	1.4	43.1	0.255	0.657
5	12.7	11.0	8.0	5.3	4.6	3.6	2.9	1.5	49.6	0.252	0.649
5	10.7	8.5	6.5	5.1	3.5	3.1	2.4	1.6	41.4	0.276	0.730
10	12.0	8.2	6.8	4.0	3.4	2.2	1.7	0.6	38.9	0.167	0.360
10	9.4	9.0	8.2	6.2	5.1	3.4	3.0	1.5	45.8	0.297	0.800
10	5.3	5.5	4.1	2.3	2.1	1.8	1.5	0.9	23.5	0.282	0.749
30	5.5	4.9	5.0	2.8	1.9	1.4	1.0	0.5	23.0	0.188	0.433
30	7.4	7.4	7.4	6.0	4.0	2.6	2.2	1.4	38.4	0.279	0.740
30	5.0	1.6	4.0	2.3	1.8	1.5	0.6	0.6	17.4	0.255	0.657

Key to table (see general key at beginning of Appendix 3)

TGF-β : nanograms TGF-β added above the Matrigel layer