Angiogenesis and Matrix Metalloproteinases in Non-Small Cell Lung Cancer

Thesis submitted for the degree of
Doctor of Medicine
at the University of Leicester

by

Dr. Giles Cox BSc. MBBS. MRCP.

Department of Oncology
University of Leicester

July 2000

UMI Number: U138670

UMI

®

Dissertation Publishing

ProQuest®

Abstract

Angiogenesis and Matrix Metalloproteinases in Non-Small Cell Lung Cancer

Dr. Giles Cox BSc. MBBS. MRCP.

The T,N,M staging system is currently the best predictor of outcome for NSCLC. The underlying tumour biology may account for wide variations in outcome between patients with similar stage disease. The aim of this study was evaluate potential invasive and metastatic pathways in order to develop a biological prognostic model for operable NSCLC.

Initially an immunohistochemical study of angiogenesis, growth factor receptors (EGFR, c-erbB-2), regulators of apoptosis (p53, Bcl-2) and matrix metalloproteinases and their inhibitors (MMP-2, MMP-9 and TIMP-2) was performed in a retrospective series of resected NSCLC tumours. Chalkley counting of CD34-immunostained microvessels was used as an indirect measure of angiogenesis. A high Chalkley count was an independent marker of poor outcome. Bcl-2 expression correlated with good prognosis suggesting that loss of Bcl-2 expression may indicate more severe molecular dedifferentiation resulting in a more aggressive phenotype. MMP-2, MMP-9 and TIMP-2 were frequently demonstrated in both tumour cells and the surrounding stroma. Tumour cell MMP-9 expression was independently associated with poor prognosis and significantly correlated with EGFR immunoreactivity. A stage-independent prognostic model using the immunohistochemical markers CD34, EGFR, MMP-9 and Bcl-2 performed on routinely processed tissue was developed. This model identified patients at particular risk of recurrence after resection who could receive adjuvant treatment with either traditional cytotoxic chemotherapy or potentially individualised therapy with more novel targeted therapeutic agents.

In vitro studies demonstrated that EGF up-regulated MMP-9 mRNA expression in 4/5NSCLC cell-lines with no effect on MMP-2, MT1-MMP, TIMP-1 or TIMP-2 mRNA expression. In association with the *in vivo* finding of a significant correlation between MMP-9 and EGFR expression, these results suggest EGFR is involved in the specific up-regulation of MMP-9 and supports the use of novel therapies including MMP inhibitors and EGFR tyrosine kinase antagonists in the treatment of NSCLC.

Acknowledgements

I am indebted to my supervisor Dr Ken O'Byrne for his constant guidance and support. His enthusiasm and commitment to improving the management and care of lung cancer patients and to developing co-ordinated cancer research in Leicester is unbounded. I will always remain grateful for the opportunity of performing research with him.

I particularly wish to thank Dr Louise Jones for her immeasurable assistance with the *in vitro* section of the thesis and the endless interpretation of the immunohistochemistry. This thesis would not have been possible without her help. I hope I can learn to be as patient with people in the face of an overwhelming workload as she is. I wish her success and continued happiness pushing the back the boundaries in breast cancer research. I must thank Sheila Dearing for teaching yet another person immunohistochemistry and for her helpful comments when nothing or everything turns brown. Linda Gordon spent hours watching over my efforts in tissue culture and never failed to have a friendly word of encouragement in scientific adversity. Thanks to my co-investigators in Denmark who helped with the c-erbB-2 studies.

Russell Leake in Oxford taught me Chalkley counting and Allen Andi shared days on the double-headed microscope. Keith Abrams assisted with the statistical analysis. George kept me sane during those interminable immuno-runs. Moral and physical support was received from all the other members of the BCRU including Afsana, Bharti, Gerald, Karen, Kufre, Matt, Suzanne, Zaffar and Zoe. The Department of Histology in Glenfield and Special Histology at the LRI supplied near-endless lung tissue and Medical Records helped trace apparently lost notes. Linda and Jenny sorted the wheat from the chaff. Thank you all.

I am grateful from the financial support from the Leicestershire Institute of Cancer Studies and a grant from the Glenfield Hospital Trust R&D fund. I also must thank all the people who generously donated cell-lines and antibodies including Rosemary Walker, Adam Patterson, Judith Coulson, Andy Wardlaw and DAKO.

Finally thanks to my wife and family who put up with the early morning meetings, late night phone calls and paucity of interesting conversation for the last two and a half years.

List of Contents

List of Figures

List of Tables

Chapter 1

Introduction:

Angiogenesis and Matrix Metalloproteinases in

Non-Small Cell Lung Cancer

Angiogenesis and matrix metalloproteinases in non-small cell lung cancer.

Background.

At the beginning of the 20th century the consensus of opinion was that lung cancer was an extremely rare disease (Alder I, 1912). Since that time the incidence has risen to such an extent that lung cancer has become one of the most common malignancies and is the leading cause of cancer related death in the Western World. During 1997, in England and Wales, there were 37,000 lung cancer deaths and the crude mortality was 80/100,000 for men and 40/100,000 for women (Office for National Statistics, 1998). This dramatic change is primarily attributable to cigarette smoking, responsible for over 85% of all cases (Boyle P, 1997). Although a reduction in cigarette smoking is beginning to produce an age-adjusted decrease in the incidence of lung cancer among men, the incidence among women is increasing, more than doubling in the USA between 1973 and 1988. As a result lung cancer has surpassed breast cancer as the commonest cause of cancer death in women in the USA. Of great concern is the upward trend in smoking in adolescents and the fact that for women in developed countries the peak of the lung cancer epidemic has not yet arrived (Boyle P, 1997)(Ginsberg RJ et al, 1997)(Wingo PA et al, 1999).

Non-small cell lung cancer (NSCLC) accounts for approximately 80% of all lung cancers and composes a heterogeneous aggregate of at least three histological sub-types including squamous cell, adeno- and large cell carcinoma. In the region of 30% of patients with NSCLC present with a tumour confined to the lung and loco-regional lymph nodes (stage I and II disease). Surgery is the treatment of choice for these patients and represents the best chance of a cure. Another 25 to 30% of patients have locally advanced disease (stage IIIa and IIIb disease). While surgical resection of the tumour may be possible in some cases, the mainstay of treatment in this patient group is palliative or radical radiotherapy. Recent studies suggest that the addition of chemotherapy may improve survival in these patients (Vokes EE, 1997). The 5 year survival for patients with stage I disease is 57-67%, stage II disease 39-55%, stage IIIa disease 9-25% and stage IIIb disease only 3-7% (van Zandwijk N et al, 1996)(Ginsberg RJ et al, 1997)(Mountain CF, 1997). Despite improvements in diagnostic imaging, surgery, radiotherapy and chemotherapy the overall survival remains appalling with only 8-14% of patients surviving 5 years after diagnosis. Novel approaches to the management of lung cancer are urgently required.

Following cellular transformation, early tumour growth is dependent on the balance between increasing cell numbers through proliferation and decreasing numbers through apoptosis and necrosis (Holmgren L et al, 1995). Potentially a small genetically unstable hypoxic tumour exposed to a hostile environment will eventually develop mutations producing a phenotype capable of resisting apoptosis and metastasizing (Harris AL, 1997). There are several steps involved in the metastatic spread of cancer from the primary tumour to a secondary remote site. Firstly the cancer cells have to escape from the primary tumour and intravasate into the blood or lymphatic circulation. The cells must survive transportation in the circulation before extravasating at a distant site. These cells then need to establish themselves at the new site before growth and replication can occur to form a metastatic colony. During this time the malignant cell has to avoid destruction by the host immune system. Vital to the growth of both the primary and metastatic disease is the capacity of the tumour to induce breakdown of the extracellular matrix (ECM) and the development of a blood supply through new blood vessel formation, a process known as angiogenesis.

The rapid growth in our knowledge of the biology of lung cancer has lead to the development of many areas of research into the treatment of this disease.

Angiogenesis.

Angiogenesis is the formation of new blood vessels from the endothelium of the existing vasculature and occurs in response to the metabolic demands of tissues and tumours. Neovascularization is a requirement for solid tumour growth beyond 1-2mm in diameter (Folkman J, 1991). Blood vessels play an important role in tumorigenesis supplying nutrients and oxygen, disposing of metabolic waste products and allowing metastatic spread.

The angiogenic process is a balance between stimulatory and inhibitory factors (table 1.1). A change that favours stimulation may trip an 'angiogenic switch' allowing the tumour to initiate the growth of new blood vessels from the surrounding host vasculature (Hanahan D et al, 1996). These regulatory factors may be endocrine, coming from the circulation, paracrine, from the adjacent tumour, stromal and inflammatory cells or extracellular matrix (ECM), and/or autocrine, from the endothelial cells themselves. Tumours promote angiogenesis by secreting or inducing the release of growth factors that stimulate endothelial cell migration and proliferation, proteolytic activity and capillary morphogenesis. There may also be inhibition or loss of the synthesis of antiangiogenic factors, such

3

as thrombospondin-1, that suppress angiogenesis (Weinstat-Saslow DL et al, 1994).

Apart from being spatially and temporally restricted, microvascular endothelial cells behave in a similar manner to invasive tumour cells. Hypoxia and hypoglycaemia induce the local activation and synthesis of pro-angiogenic cytokines and growth factors leading to changes within endothelial cells. There is an increase in adhesion molecule expression and release of proteolytic enzymes. Proteases create a gap in the basement membrane through which endothelial cells from post-capillary parent venules migrate into the perivascular stroma and form a capillary bud. Further proteolysis of the ECM produces chemotactic degradation products and releases bound growth factors. The capillary bud undergoes cell proliferation and sprout extension before maturation and lumen formation (figure 1.1). The endothelial cells in these new vascular loops are abnormal in shape and size with wide cell junctions and an irregular, leaky basement membrane. These new vessels leak plasminogen, fibrinogen and platelets leading to extravascular fibrin deposition and hypercoagulation. Inflammation plays an important part in tumorigenesis (Polverini PJ et al, 1984) with intense macrophage infiltration correlating with increased angiogenesis (Leek RD et al, 1996)(O'Byrne KJ et al, 2000).

Table 1.1 Pro-angiogenic agents.

VEGF	interleukin (IL)-1
PD-ECGF	IL-4, IL-6
TNFα	EGF
aFGF	fibrin
bFGF	integrins $\alpha_v\beta_3$, $\alpha_v\beta_5$
platelet activating factor (PAF)	MMPs
TGF-β	uPA
insulin-like growth factor-1 (IGF-1)	PGE$_1$, PGE$_2$
hepatocyte growth factor/scatter factor	angiogenin

Evaluation of angiogenesis.

The intensity of the angiogenic process in malignant tissues is measured indirectly by counting microvessels. This involves the immunohistochemical staining of vascular endothelial cells using antibodies to factor VIII, CD31 or CD34. Sections from the periphery of the tumour are used as these are usually the most vascular and there is least necrosis. Under low power three 'hot spots' are identified. These are then viewed under high power (x200-250) to acquire the microvessel density (Vermeulen PB et al, 1996). Other methods have been validated including Chalkley counting and

Figure 1.1 Mechanism of angiogenesis.

tumour cell   established vessel

ECM degradation

degradation of the ECM by
proteases released from tumour
and activated endothelial cells

angiogenic
stimulators

migration of endothelial cells
towards angiogenic stimulus
+/- proliferation leading to
formation of capillary bud

tubule formation
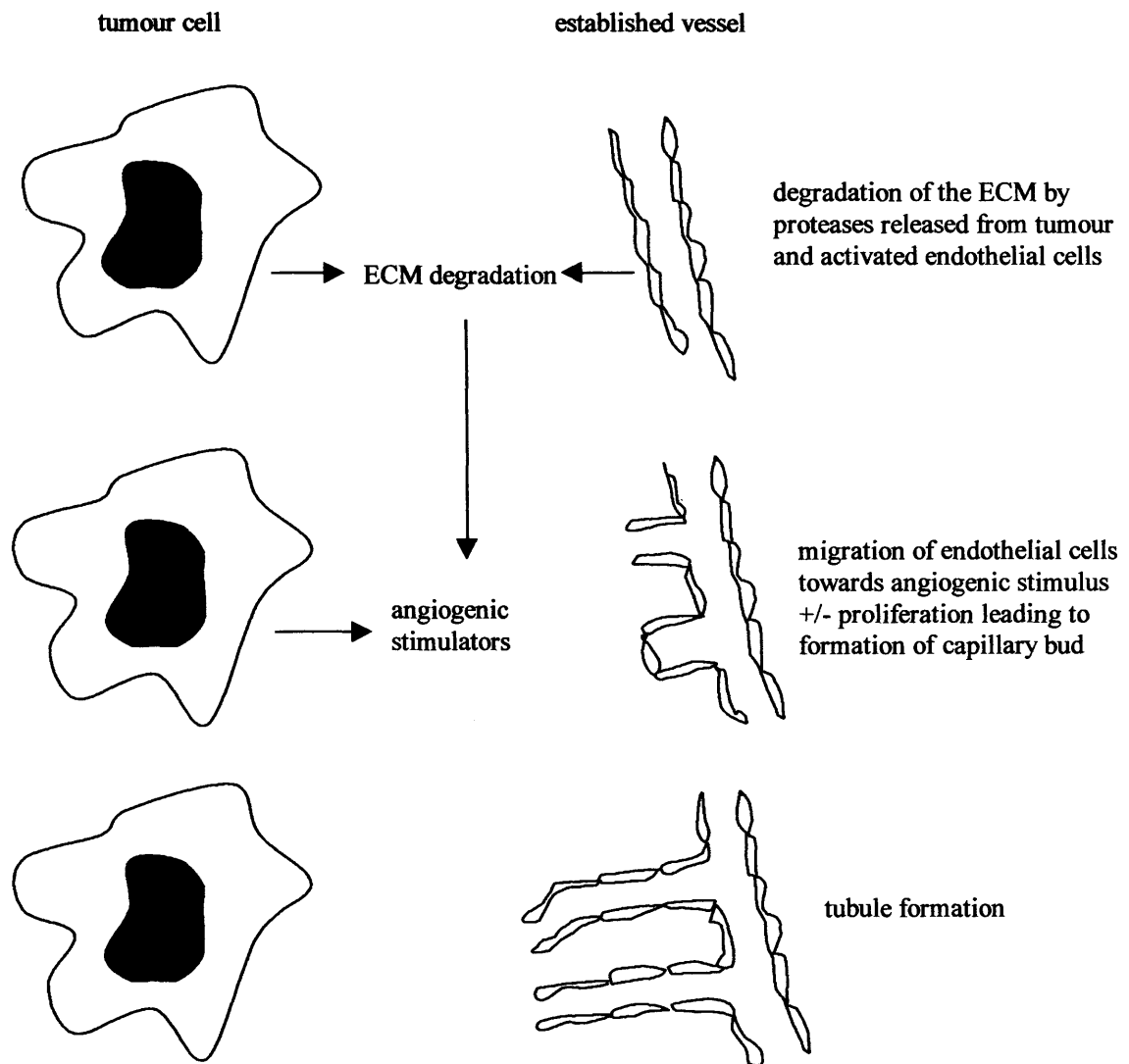
subjective eye-appraisal (Fox SB et al, 1995).

Using microvessel counting to determine the angiogenic nature of a tumour makes some important assumptions. These methods rely on the hot spots representing the angiogenic activity of the tumour and that the areas of highest vascularity are associated with the rate of metastasis. Vessel counting from a single breast tumour section has been shown to correlate with whole tumour vascularity as measured by microangiography (Martin L et al, 1997b). Microvessels are usually detected using pan-endothelial markers however these do not distinguish newly formed vessels from pre-existing vessels. As the architecture of new vessels differs from that of established vessels, the ability of tumour cells to intravasate may differ. The presence of vessels does not necessarily reflect blood-flow as this is commonly intermittent or reverse in tumours although a correlation between angiogenesis and MRI doppler blood flow has been reported (Baudu L et al, 1996) and between breast microangiography and microvessel density (Martin L et al 1997b).

Angiogenesis in non-small cell lung cancer.

Angiogenesis, measured by microvessel counting, confers a poor prognosis in many solid tumours including breast (Weidner N et al, 1991)(Toi M et al, 1993), prostate (Weidner N et al, 1993), ovarian (Hollingsworth HC et al, 1995), colorectal (Saclarides TJ et al, 1994) and gastric carcinoma (Tanigawa N et al, 1996). Many studies have associated angiogenesis with a poor prognosis in NSCLC (Macchiarini P et al, 1992)(Yamasaki K et al, 1994)(Fontanini G et al, 1995)(Angeletti CA et al, 1996)(Giatromanolaki A et al, 1996)(Harpole DH et al, 1996)(Kawaguchi T et al, 1997)(Fontanini G et al, 1997)(Matsuyama K et al, 1998)(Duarte IG et al, 1998)(Aikawa H et al, 1999)(Dazzi C et al, 1999)(Yano T et al, 2000). A number of studies suggest adenocarcinomas may have higher vessel counts than squamous cell carcinoma. This may account for the increased likelihood of metastases with this histological sub-type (Yuan A t al, 1995)(Sikora J et al, 1997). Evidence that angiogenesis is associated with the metastatic spread of tumour cells is supported by the finding that a high microvessel count is associated with increased peri-operative shedding of breast cancer cells (McCulloch P et al, 1995).

Some recent studies in NSCLC have not found microvessel count to be a predictor of survival (Pastorino U et al, 1997)(Apolinario RM et al, 1997)(Chandrachud LM et al, 1997)(Decaussin M et al, 1999). A number of factors may account for the findings in these studies. The sensitivity of microvessel counting is dependent upon the experience of the investigators (Vermeulen PB et al,

1997). Several studies have shown a correlation between microvessel count and stage therefore studying single stage disease may lead to a negative result (Fontanini G et al, 1995). Furthermore a recent study of NSCLC tumours has demonstrated heterogeneity of vasculature even within blocks from the same region. This study also showed that the periphery of the tumour does not always contain the highest number of vessels. These intra-tumour variations may account for some of the contradictory prognostic results (Schor AM et al, 1998). Tumours with an 'alveolar pattern', where there is no parenchymal destruction, no obvious intratumoral angiogenesis and the alveolar septae are still present, appear to have a worse prognosis than tumours with an 'angiogenic pattern'. This suggests that an existing suitable vascular bed may be exploited without the need for new vessel proliferation (Pezzella F et al, 1997).

Bronchial dysplasia and carcinoma *in situ* have increased vascularity compared to normal bronchial epithelium and hyperplasia (Fontanini G et al, 1996)(Fisseler-Eckhoff A et al, 1996). A similar pattern is also found in preneoplastic cervical (Dobbs SP et al, 1997) and breast (Guidi AJ et al, 1994) lesions. Using fluorescent bronchoscopy, angiogenic squamous dysplastic lesions have been identified in 34% of high-risk smokers without carcinoma and in 60% of patients with squamous cell lung carcinoma (Keith RL et al, 2000). These lesions consist of a capillary blood vessel closely juxtaposed to and projecting into metaplastic or dysplastic squamous bronchial epithelium. These lesions are also associated with marked proliferation. Together these findings suggest angiogenesis is an early event in the malignant process.

Angiogenic growth factors.

Growth factors play a crucial role in angiogenesis and malignancy (figure 1.2). For angiogenesis to occur there must be a net balance in favour of angiogenic inducers over inhibitors. A large number of growth factors and cytokines have been shown to stimulate angiogenesis (Bouck N et al, 1996) and to induce the secretion of other growth factors (Tsai J-C et al, 1995). Growth factors released from the activated endothelium may produce an autocrine effect on itself and a paracrine effect on the associated neoplastic cells. Growth factors secreted by tumour, inflammatory and stromal cells bind to soluble growth factor receptors in the stroma (Hannekan A et al, 1995) where they can be released by proteolytic enzymes secreted by an invading tumour. Growth factors may be chemotactic and mitogenic, targeting kinase receptors expressed by endothelial and tumour cells. Tumour cell growth factor production will lead to self-proliferation and indirectly mediate growth by stimulating tumour

Figure 1.2 Growth factors and the induction of angiogenesis.
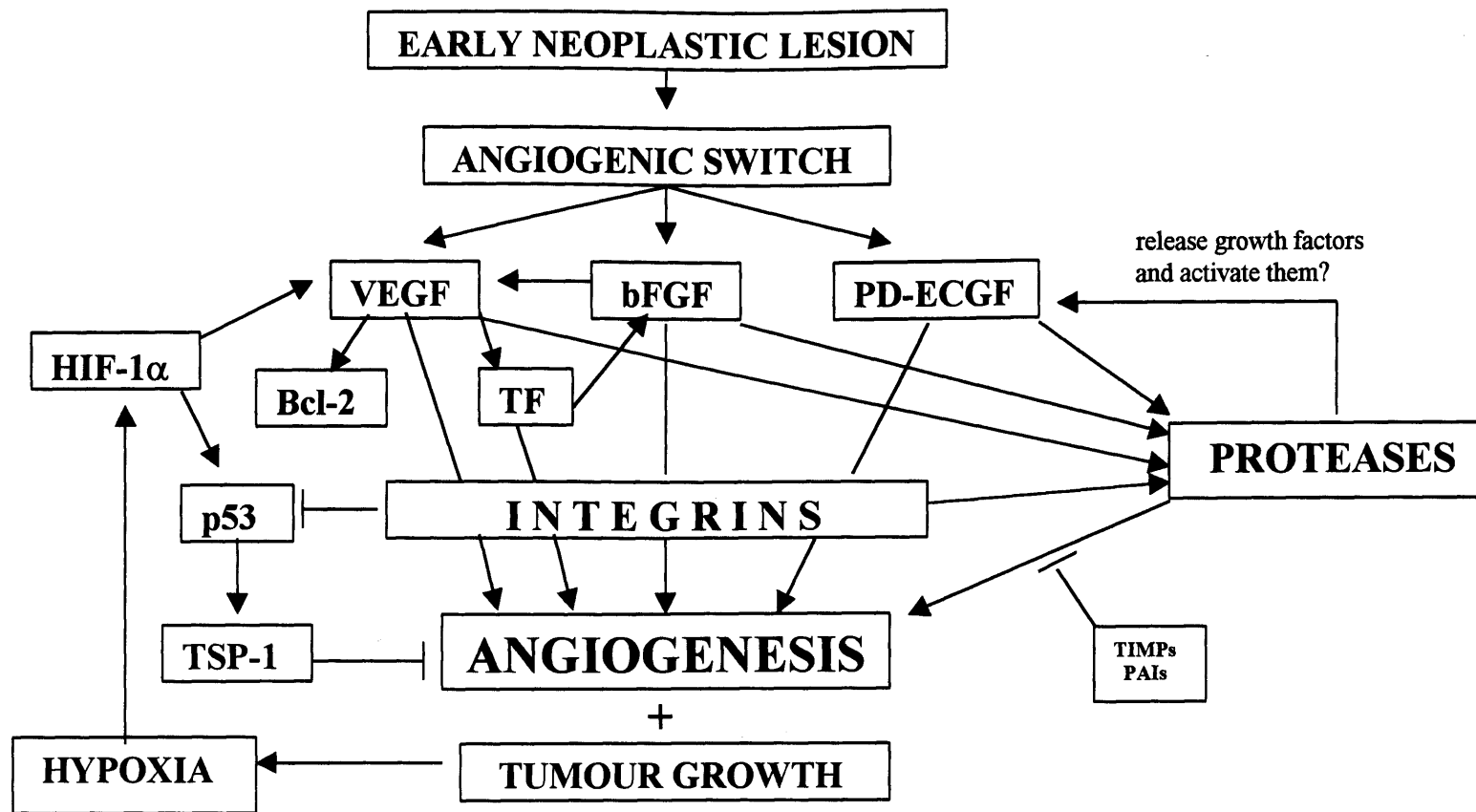
Key: VEGF(vascular endothelial growth factor), bFGF (basic fibroblast growth factor), PD-ECGF (platelet-derived endothelial cell growth factor), TF (tissue factor), HIF-1α (hypoxia-inducible factor-1α), TSP-1 (thrombospondin-1), TIMPs (tissue inhibitors of metalloproteinases), PAIs (plasminogen activator inhibitors), GFs (growth factors) and ECM (extracellular matrix).

vessel proliferation. Growth factors may also up-regulate the production of plasmin activators and MMPs. Studies have shown growth factors such as transforming growth factor-α (TGFα) and transforming growth factor-β (TGFβ) to have opposing effects *in vitro* and *in vivo* (Frater-Schroder M et al, 1987) suggesting a complicated interaction that may depend on their relative concentrations or the tissue type upon which they are acting. Serum (Kondo S et al, 1994) and urine (O'Brien TS et al, 1995) measurements of angiogenic factors are higher in tumour patients than controls and their role as tumour markers or surrogate markers of angiogenesis is currently under evaluation.

Several growth factors have been investigated in NSCLC. This review will concentrate on the growth factors that have been studied most closely in relation to angiogenesis in this disease

Vascular endothelial growth factor family

The vascular endothelial growth factor (VEGF) family consists of VEGF, placental growth factor and VEGF-B, -C, -D and -E. They are all dimeric glycoproteins with homologous amino acid sequences shared with PDGF-α and -β and bind to similar tyrosine kinase receptors (Conn G et al, 1990). VEGF is found in a number of isoforms created by differential splicing of RNA (Houck KA et al, 1991)(Tischer E et al, 1991). VEGF$_{165}$ is a 45kDa glycoprotein and the most abundant isoform. VEGF$_{189}$ and VEGF$_{206}$ are more basic and remain close to the membrane strongly bound to heperan sulphate. VEGF$_{121}$ is more acidic and the most soluble. VEGF binds with high affinity to two tyrosine kinase receptors, VEGFR-1 (Flt-1) (deVries C et al, 1992) and VEGFR-2 (Flk-1/KDR) (Terman BI et al, 1992). Ligand binding causes receptor dimerization, autophosphorylation and signal transduction.

VEGF is the most potent and specific growth factor for endothelial cells. Hypoxia is a common finding in tumours. Hypoxia increases VEGF expression (Schweiki D et al, 1992) by inducing VEGF transcription via hypoxia-inducible factor-1α (Semenza GL, 1996) and by stabilizing VEGF mRNA (Levy AP et al, 1996). Hypoxia also increases the synthesis of VEGF receptors (Waltenberger J et al, 1996). The growth factors bFGF, epidermal growth factor (EGF) and PDGF all stimulate VEGF secretion (Tsai J-C et al, 1995). The pro-inflammatory cytokine interleukin (IL)-1β enhances VEGF expression (Li J et al, 1995) whilst interleukin-12, an anti-inflammatory cytokine, down-regulates VEGF expression *in vivo*. Wild-type p53 down-regulates VEGF promoter activity (Mukhopadhyay D et al, 1995) whilst mutant p53 has the opposite effect (Keiser A et al, 1994).

Vascular permeability is increased by VEGF allowing the leakage of plasma proteins and fibrin deposition into the ECM (Dvorak HF et al, 1995). Fibrin will act as a scaffold for vascular structures. VEGF induces tissue factor synthesis (Claus M et al, 1996) leading to thrombin production from prothrombin. This in turn stimulates progelatinase A (proMMP-2) activation and endothelial cell proliferation (Zucker S et al, 1998). VEGF also induces urokinase plasminogen activator (uPA), tissue plasminogen activator (tPA), plasminogen activator inhibitor-1 (PAI-1) (Pepper MS et al, 1992a) and interstitial collagenase (Unemori EN et al, 1992) expression allowing ECM degradation. These effects facilitate the development of the capillary bud.

The coagulation and fibrinolysis pathways may also involve VEGF. Platelets transport VEGF in the circulation (Verheul HMW et al, 1997) and release VEGF when they are activated (Mohle R et al, 1997). Platelet adherence to tumour vessels may be increased by low blood-flow in tumours and/or the increased presentation of platelet-binding proteins either through stromal remodelling, microvessel hyperpermeability or the release of von Willebrand factor (vWF). Platelet adherence and possible extravasation results in platelet activation with the release of angiogenesis regulation factors including VEGF. This resultant increase in the local concentration of VEGF may lead to the induction of angiogenesis (Pinedo HM et al, 1998).

There is considerable evidence that VEGF plays an important role in tumour associated angiogenesis. Transfection of VEGF cDNA leads to an increase in tumour growth, vascularity and metastases (Senger DR et al, 1994) and this effect can be inhibited using VEGF antibodies (Kondo S et al, 1993)(Zhang HT et al, 1995). Mutation of the VEGF receptor VEGFR-2 (Flk-1/KDR) inhibits angiogenesis (Millauer B et al, 1994) and gene knock-out studies suggest a role for the receptors VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1/KDR) in neovascularization (Shalaby F et al, 1995)(Fong G-H et al, 1995). Tumours may also express VEGF receptors as VEGF antibodies block VEGF-induced choriocarcinoma cell proliferation (Charnock-Jones DS et al, 1994). Inactivation of VEGFR-2 (Flk-1/KDR) using specific antibodies decreases angiogenesis and malignant invasion without reducing tumour cell proliferation changing a malignant phenotype into a benign phenotype *in vivo* (Skobe M et al, 1997). VEGF also inhibits cell death by apoptosis after ionizing radiation (Katoh O et al, 1995) and appears to induce the expression of Bcl-2 in endothelial cells (Nor JE et al, 1999). The ability of cells to produce VEGF and therefore resist apoptosis may tip the balance in favour of proliferation and growth.

In the lung VEGF is expressed by normal bronchiolar and differentiated columnar epithelial cells and by alveolar macrophages. Stromal fibroblasts and macrophages are only occasionally positive. VEGF tumour immunostaining is usually cytoplasmic especially in the perinuclear region. High levels of expression of VEGF and their receptors are found in many solid tumours including breast (Brown LF et al, 1995), colon (Brown LF et al, 1993a), ovary (Olson TA et al, 1994), cervix (Guidi AJ et al, 1995), kidney (Brown LF et al 1993b) and non-small cell lung carcinoma (Mattern J et al, 1995)(Holm C et al, 1996)(Ohta Y et al, 1996)(Takanami I et al, 1997a). VEGF expression in breast cancer (Relf M et al, 1997) and NSCLC (Mattern J et al, 1995)(Ohta Y et al, 1996)(Takanami I et al 1997a)(Fontanini G et al, 1997b)(Ohta Y et al, 1997) and VEGFR-2 (Flk-1/KDR) expression in advanced colon carcinoma (Takahashi Y et al, 1995) are associated with a poor prognosis. There is a correlation between microvascular density and VEGF expression in NSCLC (Fontanini G et al, 1997b)(Mattern J et al, 1996)(Imoto H et al, 1998)(Tsao MS et al, 1997)(Yano T et al, 2000) and also in breast (Toi M et al, 1995a), cervical (Guidi AJ et al, 1995) and gastric cancer (Maeda K et al, 1996). However not all immunohistochemical studies in NSCLC have demonstrated this association (Decaussin M et al, 1999). Employing reverse transcription-polymerase chain reaction (RT-PCR) techniques, $VEGF_{121}$, $VEGF_{165}$ and $VEGF_{189}$ have been found to be associated with angiogenesis and/or a poor prognosis in NSCLC (Oshika Y et al, 1998)(Fontanini G et al, 1999). The association of VEGF expression with increased microvascular density enhances the evidence supporting a role for VEGF as a mitogenic growth factor for vascular endothelial cells in malignant disease.

A recent study has shown that the VEGF/KDR activated microvessel density was significantly higher in the invasive front of a tumour and in the normal lung adjacent to the tumour compared to inner tumour areas and distant normal lung (Koukourakis MI et al, 2000). The study concluded that intense VEGF/KDR angiogenic pathway activation is a feature of more than half of NSCLC tumours and is associated with a poor outcome.

Platelet derived-endothelial cell growth factor

Platelet-derived endothelial cell growth factor (PD-ECGF) is an angiogenic factor secreted from platelets. PD-ECGF is the non-heparin-binding 90kDa homodimer thymidine phosphorylase (TP) (Moghaddam A et al, 1992). TP increases DNA synthesis, endothelial cell migration *in vitro* and tumour growth and angiogenesis *in vivo* (Moghaddam A et al, 1995). Transfection of the TP gene

into transformed fibroblasts in nude mice leads to an increase in tumour vascularity (Ishikawa F et al, 1989). The presence of TP in tumours may be important in the choice of treatment. 5-fluorouracil (5-FU) is activated to fluorodeoxyuridine by TP (Sotos GA et al, 1994) and raised intracellular TP levels increase fluoropyridimine chemosensitivity (Patterson A et al, 1995). The addition of interferon-α to cancer cells increases both TP activity and 5-FU cytotoxicity (Schwartz EL et al, 1994).

Patients with malignancy have raised serum TP levels (Pauly JL et al, 1977). PD-ECGF is over-expressed in solid tumours (Yoshimura A et al, 1990). A correlation between PD-ECGF expression and microvascular density has been shown in some breast cancer studies (Toi M et al, 1995b)(Relf M et al, 1997) but not in others (Fox SB et al, 1996). TP expression is invariably seen in the alveolar macrophages of normal lung. Weak PD-ECGF immunostaining occurs in bronchial basal cells and well differentiated columnar cells. Weak immunoreactivity is also seen in stromal fibroblasts. NSCLC cell-lines that express PD-ECGF tend to be well-differentiated (Heldin NE et al, 1993). PD-ECGF expression both in tumour cells and in stromal fibroblasts correlates with angiogenesis (Koukourakis MI et al, 1998). Fibroblast PD-ECGF expression is found especially in areas of abundant stroma. This suggests that the growth factor may be a marker of an active remodelling stroma. Focal PD-ECGF over-expression is associated with locally raised vessel counts in both high and low angiogenic tumours. Stromal fibroblast expression may be induced by macrophage infiltration (Giatromanolaki A et al, 1998a). However not all studies report a positive correlation between PD-ECGF and angiogenesis in NSCLC (Aikawa H et al, 1999).

Basic fibroblast growth factor

The single basic fibroblast growth factor (bFGF) gene encodes for multiple bFGF isoforms between 18-24kDa in a similar way to VEGF. The cytokines interleukin-2 and interferon-α (Cozzolino F et al, 1993) and the coagulation factor thrombin all up-regulate bFGF production (Weich H et al, 1991). Secreted bFGF adheres to the ECM where it is released by proteolytic enzymes (Vlodavsky I et al, 1991a). bFGF induces protease expression (Gualandris A et al, 1994) and also modulates the expression of integrins (Klein S et al, 1993). bFGF is proangiogenic *in vitro* and leads to the formation of capillary-like structures in collagen cells (Montesano R et al, 1986). *In vivo* studies also demonstrate bFGF to be angiogenic (Gualandris A et al, 1994) and this effect can be inhibited using bFGF antibodies (Hori A et al, 1991). bFGF stimulates VEGF secretion (Tsai J-C et al, 1995) and

12

the effects of VEGF and bFGF appear to be synergistic *in vitro* (Pepper MS et al, 1992b)(Goto F et al, 1993)(Stavri GT et al, 1995) and *in vivo* (Ashara T et al, 1995). Upregulation of a secreted bFGF binding protein (FGF-BP) increases tumour growth and angiogenesis (Czubayko F et al, 1994). FGF-BP mRNA is elevated in squamous cell carcinoma (Czubayko F et al, 1994). It has been proposed that upregulation of FGF-BP in tumours leads to increased secretion of FGF-BP protein into the ECM. This adheres to the bFGF that is bound to heperan sulphate and releases soluble active bFGF into the ECM (Czubayko F et al, 1997).

Many solid tumours demonstrate increased expression of bFGF and its receptor including malignant melanoma (Becker D et al, 1989), ovarian carcinoma (Crickard K et al, 1994) and NSCLC (Takanami I et al, 1996)(Takanami I et al, 1997). bFGF has been associated with poor prognosis in NSCLC, the expression of the growth factor and its receptor FGFR-1 correlating with microvessel density (Taknami I et al, 1997). However these observations were not supported by the findings of a recent study in which bFGF stromal cell expression was inversely correlated with tumour stage and no correlation was seen between either bFGF tumour or stromal expression and microvessel count (Guddo F et al, 1999).

Other growth factors

There are several other growth factors thought to be involved in angiogenesis in NSCLC. Hepatocyte growth factor (HGF) is a highly angiogenic cytokine produced from epithelial and endothelial cells. Raised HGF levels in tumours are associated with a significantly worse outcome in NSCLC, especially in early stage disease (Siegfried JM et al, 1998). Tissue factor (TF) is the primary initiator of coagulation and is involved in intracellular signalling and tumour angiogenesis (Zhang Y et al, 1994). Tissue factor expression correlates with angiogenesis, survival and VEGF expression in NSCLC (Koomagi R et al, 1998). Heparin-binding growth-associated molecule (HB-GAM) is functionally similar to the fibroblast growth factor family. HB-GAM acts both as a transforming growth factor and an angiogenic promoter in some NSCLC cell-lines (Jager R et al, 1997). The CXC chemokine family is composed of chemotactic molecules of less than 10kDa. Members containing an ELR motif have potent angiogenic activity and those without the ELR motif are angiostatic. Interleukin-8 and epithelial-neutrophil activating peptide (ELR-CXC chemokines) are angiogenic in NSCLC (Arenburg DA et al, 1997)(Arenburg DA et al, 1998) whereas interferon-γ-inducible protein 10 (a non-ELR-CXC chemokine) leads to angiostasis (Arenburg DA et al, 1997).

Growth factor receptors.

The erb/HER type I family of tyrosine kinase receptors are involved in cell proliferation, motility and differentiation. Ligands for the erb receptors include EGF, TGFα, β-cellulin and amphiregulin. Ligand binding causes dimerization with another erb/HER type I receptor. This causes activation of an intracellular tyrosine kinase domain and transmission of growth signals triggering DNA synthesis (Walker RA, 1998). Two important members of the erb/HER type I receptor family are epidermal growth factor receptor (EGFR) and c-erbB-2 (neu or HER-2). It has been postulated that the erb receptors are the 'master switches' of metastasis as they appear to play a role in many metastatic pathways (Eccles SA, 2000). Actin cytoskelton-linked erb receptors may assist cell migration (Wells A et al, 1998). Cells that overexpress erb receptors show aberrant cell-cell and cell-matrix interactions mediated through altered integrin and cadherin function (Genersch E et al, 1996). EGFR and c-erbB-2 signalling can upregulate the expression of specific MMPs enhancing invasion (O-charoenrat P et al, 2000b). Finally activated erb oncogenes can increase tumour cell adhesion to endothelial cells and upregulate VEGF. This could potentially facilitate angiogenesis and vascular invasion (Petit AM et al, 1997).

EGF-like ligands

Coexpression of EGFR and EGF-like ligands has been associated with lower overall and relapse-free survival in NSCLC (Kostyleva OI et al, 1999). Enhanced expression of amphiregulin has been found to correlate with both c-erbB-2 expression and poor outcome (Fontanini G et al. 1998a). No study has demonstrated a prognostic role for TGFα (Rusch V et al, 1997)(Fontanini G et al, 1998a). EGF and TGFα are two of the most potent inducers of VEGF and are therefore potential stimulators of angiogenesis (Tsai J-C et al, 1995). There are no published studies evaluating the association between EGFR ligand expression and angiogenesis in NSCLC.

Epidermal growth factor receptor

EGFR expression is generally low in normal bronchial epithelium and is enhanced in preneoplastic and neoplastic lesions especially squamous cell carcinomas (Hendler FJ et al, 1984). EGFR has been shown to be associated with poor prognosis in NSCLC in some studies (Veale D et al, 1993)(Volm

M et al, 1993)(Harpole DH et al, 1996)(Pastorino U et al, 1997)(Ohsaki Y et al, 2000) but not in others (Pfeiffer P et al, 1996)(Rusch V et al, 1997)(Fontanini G et al, 1998a). Inhibition of EGFR with antibodies or tyrosine kinase inhibitors inhibits tumour growth and metastases at least in part by decreasing angiogenesis secondary to down-regulating VEGF, IL-8 and bFGF (Bruns CJ et al, 2000). However EGFR expression has not been shown to correlate with microvessel counts in NSCLC tumours (Pastorino U et al, 1997)(Fontanini G et al, 1998a).

c-erbB-2

Membranous staining for c-erbB-2 has been demonstrated in 2-40% of NSCLC tumours (Kern JA et al, 1990)(Pastorino U et al, 1997)(Diez M et al, 1997) and found to be associated with a poor prognosis for adenocarcinoma in one of these studies (Pfieffer P et al, 1996). Although only membranous expression of c-erbB-2 would be expected to be functionally important, several studies have shown cytoplasmic c-erbB-2 expression, especially in adenocarcinoma, to be associated with a poor prognosis (Tateishi M et al, 1991)(Harpole DH et al, 1996)(Giatromanolaki A et al, 1996a). ELISA quantification of c-erbB-2 protein levels in NSCLC tumour samples shows a worse prognosis as levels increase (Diez M et al, 1997). c-erbB-2 expression has been shown to be inversely correlated with vessel counts in one recent study (Koukourakis MI et al, 1999) but not in another (Fontanini G et al, 1998a).

Apoptosis.

Neoplastic transformation is brought about through the activation of oncogenes or the inactivation of tumour suppressor genes. Lung cancer is associated with the expression of several of these genes including p53, Bcl-2, *myc*, *ras* and retinoblastoma gene protein (Rb) (Sekido Y et al, 1998). The rate of tumour growth is defined by the balance between cell proliferation on the one hand and cell loss due to necrosis and apoptosis (programmed cell death) on the other. Successful tumour therapies largely rely on an induction of the endogenous apoptotic pathways (Hickman JA et al, 1995).

p53

An alteration in either the p53 gene or protein is the most common change in human malignancy. The p53 gene encodes a 393 amino acid nuclear phosphoprotein that has a pivotal role regulating cell proliferation and apoptosis. p53 causes arrest of the cell cycle at the G_1-phase, increases DNA repair

time, decreases replication DNA synthesis and induces apoptosis. p53 induced apoptosis is triggered by DNA damage, cytokines, growth factor-deprivation (Canman CE et al, 1995) and hypoxia (Graeber TG et al, 1994). Wild-type p53 decreases VEGF expression (Mukhopadhyay D et al, 1995) whilst mutant p53 increases protein kinase C induced VEGF expression (Keiser A et al, 1994). Mutations of the p53 gene enable a cell to survive longer under hypoxic conditions (Graeber TG et al, 1996). Loss of p53 expression leads to increased angiogenesis by decreasing the 170kDa matrix glycoprotein thrombospondin-1 (Dameron KM et al, 1994).

Thrombospondin-1 modulates platelet aggregation, wound healing and cell adhesion. It inhibits angiogenesis partly through an effect on the proteolytic activity of the endothelium (Good DJ et al, 1990). Alternative splicing and post-transcriptional modification leads to differing effects (Good DJ et al, 1993). Tumour angiogenesis is associated with decreased thrombospondin-1 expression in various tumour types and transfection with thrombospondin-1 cDNA decreases angiogenesis (Weinstat-Saslow DL et al, 1994)(Zabrenetzky V et al, 1994). Recent evidence suggests that thrombospondin-1 mediates endothelial cell apoptosis and inhibits angiogenesis in association with increased expression of Bax, decreased expression of Bcl-2 and caspase-3 activation (Nor JE et al, 2000).

As p53 has a short half-life, immunostaining for p53 identifies protein over-expression or more often a stable mutant protein. p53 expression is found in pre-invasive lesions of the bronchial tree suggesting mutation is an early event in the process of malignant transformation leading to lung cancer (Sozzi G et al, 1992)(Bennett WP et al, 1993). The evidence of a prognostic role for p53 expression in NSCLC is contradictory. p53 expression has been shown to be related to either a good prognosis (Lee JS et al, 1995)(Passlick B et al, 1995)(Top B et al, 1995), a poor prognosis (Quinlan DC et al, 1992)(Horio Y et al, 1993)(Harpole DH et al, 1995)(Dalquen P et al, 1996)(Fukuyama Y et al, 1997)(Fontanini G et al, 1997c) or to have no prognostic significance (McLaren R et al, 1992)(Nishio M et al, 1996). Similarly p53 expression has been shown to correlate with angiogenic activity (Fontanini G et al, 1997c) and to have no correlation with angiogenic activity (Giatromanolaki A et al, 1996a). As p53 tightly regulates thrombospondin-1, a positve correlation between mutant p53 expression and angiogenesis would be expected. It is possible that any regulating role of p53 or p21 on angiogenesis may be in tandem with other oncogenes eg c-erbB-2 (Giatromanolaki A, 1996b). Recent studies suggest a correlation between nuclear p53 and VEGF expression in NSCLC (Fontanini G et al, 1997c)(Fontanini G et al, 1998b). An inverse correlation

between VEGF expression and cytoplasmic/perinuclear p53, a feature suggestive of wild-type p53 expression, has recently been shown (Giatromanolaki A et al, 1998b).

Bcl-2

Bcl-2 is a member of a family of genes involved in the regulation of apoptosis. The Bcl-2 gene differs from conventional oncogenes as it can neither promote growth nor directly lead to cellular transformation. Raised Bcl-2 levels protect cells from the induction of apoptosis by wild-type p53 (Chiou S-K et al, 1994) whilst p53 down-regulates Bcl-2 gene-expresion (Miyashita T et al, 1994). Cells that are co-transfected to increase Bcl-2 and c-*myc* expression prevent the transmembrane passage of p53 from the cytoplasm to the nucleus during G_1 phase (Ryan JJ et al, 1994). Increased Bcl-2 expression prevents cell death from a wide range of insults including growth factor depletion, ionizing irradiation and chemotherapeutic regimes (Nunez G et al, 1990)(Miyashita T et al, 1992)(Lotem J et al, 1993).

Bcl-2 immunostaining is seen in the basal cells of normal bronchial epithelium (Koukourakis MI et al, 1997b) where it may assist the survival of stem cells. Bcl-2 expression has been demonstrated in breast (Bhargava V et al, 1994), prostatic (McDonnell TJ et al, 1992), gastrointestinal (Ayhan A et al, 1994) and lung carcinomas (Pezzella F et al, 1993)(Fontanini G et al, 1995b)(Ohsaki Y et al, 1996)(Pastorino U et al 1997)(Fleming MV et al, 1998). The association of Bcl-2 immunopositivity and prognosis in NSCLC remains controversial. Several studies have shown a good prognosis (Pezzella F et al, 1993)(Fontanini G et al, 1995b)(Koukourakis MI et al, 1997b)(Pasorino U et al, 1997)(Laudanski J et al, 1999) whilst others have shown no survival advantage (Anton RC et al, 1997)(Fleming MV et al, 1998). This may be due partly to inconsistent or ill-defined criteria for Bcl-2 positivity. A recent study has suggested that Bcl-2 is capable of altering the expression of MMPs and TIMPs enhancing migration and invasion of tumour cells (Wick W et al, 1998). Bcl-2 may therefore have other actions in addition to a role in apoptosis.

Two studies demonstrated an inverse relationship between Bcl-2 expression and angiogenesis (Koukourakis MI et al, 1997b)(Fontanini G et al, 1998b) suggesting the improved prognosis in Bcl-2 expressing tumours may be due to low vascularization.

Some studies have shown a correlation between p53 and Bcl-2 expression in NSCLC (Pezzella F et

al, 1993)(Walker C et al, 1995)(Fontanini G et al, 1995a) whilst others have not (Apolinario RM et al, 1997)(Koukourakis MI et al, 1997b)(Fontanini G et al, 1998b). Any regulatory role of Bcl-2 on angiogenesis must therefore take place through pathways that do not necessarily include p53. Bcl-2 may also enhance the production of an angiogenic inhibitor (Koukourakis MI et al, 1997b). One study in NSCLC shows apoptosis occurring independent of Bcl-2 and p53 over-expression (O'Neill AJ et al, 1996). The effects of Bcl-2 and p53 may therefore be blocked by other oncogene products or regulators of apoptosis such as IGF-1 (O'Neill AJ et al, 1996) (figure 1.3).

Adhesion and angiogenesis.

Endothelial cells adhere to each other and to the ECM during migration and capillary tube formation. There are four adhesion molecule families including the cadherins, selectins, integrins and the immunoglobulin super-family. The adhesion molecule profile of a cell has a marked influence on its behaviour.

E-cadherin is a calcium-dependent transmembrane adhesion molecule involved with alpha-catenin, beta-catenin and plakoglobin in the formation of cadherin-catenin cell-cell adherens junctions. Dysfunction of these junctions may increase the ability of a tumour cell to invade and metastasise. E-cadherin expression is inversely associated with both stage and lymph node spread in NSCLC (Sulzer MA et al, 1998)(Shibanuma H et al, 1998). A high MMP-2 and -9:E-cadherin gene ratio has been associated with increased risk of relapse after resection of stage I NSCLC (Herbst RS et al, 2000). The decrease in E-cadherin expression correlates with a reduction in expression for alpha-catenin (Shibanuma H et al, 1998)(Toyoyama H et al, 1999) and beta-catenin (Retera JM et al, 1998). Beta-catenin immunostaining in NSCLC is inversely correlated with lymph node spread and prognosis (Retera JM et al, 1998).

E-selectin is an endothelial membrane glycoprotein specifically expressed or secreted by activated endothelial cells. E-selectin may enhance tumour angiogenesis and the adhesion of tumour cells at a distant site. Antibodies to E-selectin decrease the formation of capillary-like tubes *in vitro* (Nguyen M et al, 1993).

Integrins are a family of transmembrane proteins involved in cell/cell and cell/matrix adhesion. They are involved in cellular anchorage, migration and signalling. The binding of ECM proteins to

Figure 1.3 p53, Bcl-2, apoptosis and angiogenesis.
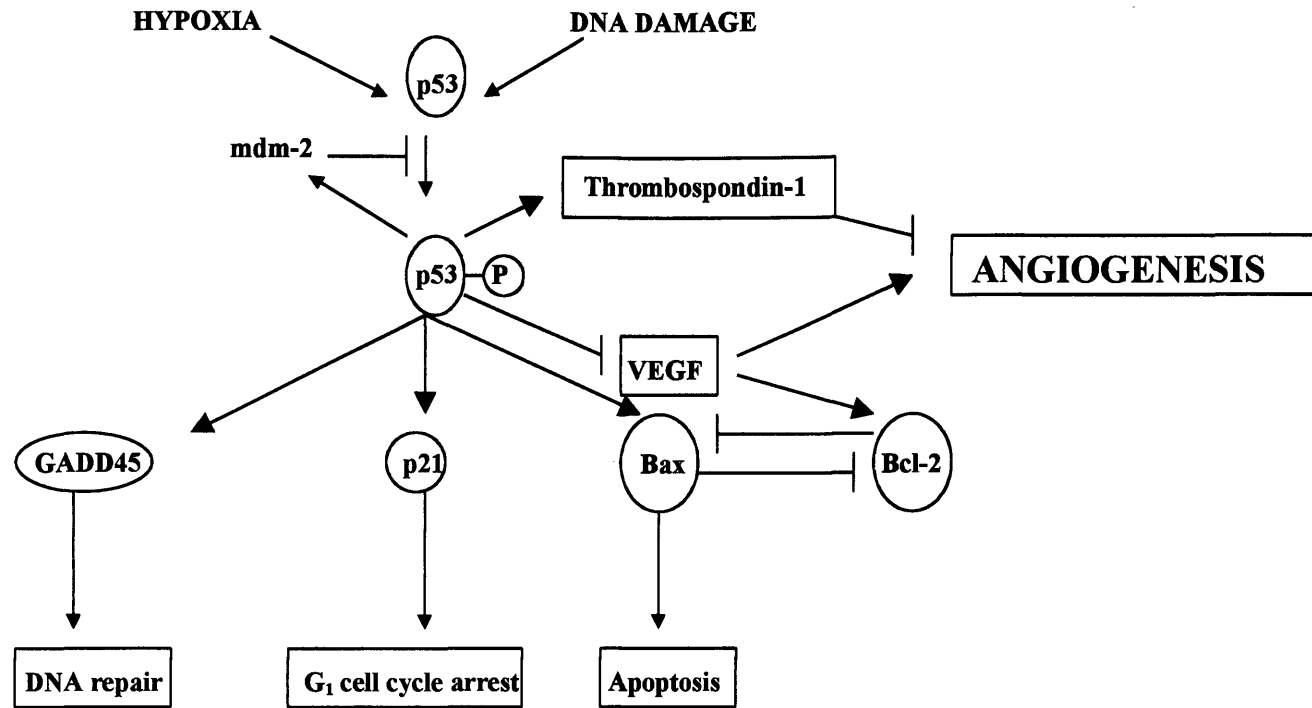
Wild-type p53 inhibits angiogenesis whilst p53 downregulation or mutation promotes angiogenesis.

integrins alters gene expression and plays an important role in cellular proliferation and differentiation. Integrins are made up of more than 20 combinations of non-covalently linked α and β chains and differing combinations are expressed by different cell-types. They are essential for migration and capillary tube formation *in vitro* (Luscinskas FW et al, 1994). Integrins and growth factors may collaborate in the phosphorlyation of tyrosine kinase receptors and have been implicated in the activation of the mitogen-activated protein (MAP) kinase pathway (Miyamoto S et al, 1996).

The integrin $\alpha_v\beta_3$ is closely linked with angiogenesis. It is expressed in growing vessels and is upregulated in response to pro-angiogenic molecules such as $TNF\alpha$, bFGF (Brooks PC et al, 1994) and VEGF (Suzuma L et al, 1998). $\alpha_v\beta_3$ binds gelatinase A (MMP-2) and will localize proteolysis to the cell edge (Brooks PC et al, 1996). Antibodies to $\alpha_v\beta_3$ decrease blood vessel growth in chick chorioallantoic membrane assays (Brooks PC et al, 1994). The expression of $\alpha_v\beta_3$ during angiogenesis in endothelial cells leads to suppression of p53 activity and therefore p21 activity as well as increasing the bcl-2/bax ratio. The outcome of this is to decrease apoptosis (Stromblad S et al, 1996). Loss of α_v-integrin expression has been shown to be associated with high levels of recurrence for node-negative NSCLC (Smythe WR et al, 1997).

Integrins containing the β_1 chain bind to ECM components including fibronectin, laminin and type IV collagen. Proteolysis of the ECM will release these components allowing them to bind to the β_1 chain and induce signal transduction. The β_1 chain is involved in the expression of several MMPs including interstitial collagenase (MMP-1), stromelysin-1 (MMP-3) and gelatinase B (MMP-9) (Huhtala P et al, 1995). Monoclonal antibodies against the β_1 subunit block the formation of endothelial cords *in vitro* (Bauer J et al, 1992).

Cyclooxygenases and angiogenesis.

The cyclooxygenases COX-1 and COX-2 catalyse the first two steps in prostaglandin synthesis. COX-1 activity is constitutively expressed in nearly all cell types and plays a central role in many normal physiological processes through the synthesis of prostacyclin and prostaglandins. COX-2 is rarely expressed in normal tissues but is induced in response to inflammatory stimuli. These stimuli include the pro-inflammatory cytokines IL-1β, IL-2, and TNF-α. The precise prostaglandin synthesised as a result of COX-2 induction depends on the specific synthase enzyme(s) present in the cell. For example PGE_2 has been implicated in the pathogenesis of a number of malignant

processes and is synthesised by prostaglandin E_2 synthase (O'Byrne KJ et al, 2000b).

Over-expression of COX-2 and/or PGE_2 is seen in a range of malignant diseases including those arising from the lung (Wolff H et al, 1998). A number of studies have clearly demonstrated that non-steroidal anti-inflammatory drugs (NSAIDs) and specific COX-2 inhibitors have an anti-proliferative activity against solid tumours both *in vitro* and *in vivo*. They also prevent haematogenous spread of tumours provided the malignancy over-expresses COX-2 (Molina MA et al, 1999)(Tomozawa S et al, 1999).

Recent work has shown that angiogenic growth factor-induced angiogenesis in the rat sponge implant model is associated with upregulation of COX-2 in the stromal tissues. Furthermore COX-2 inhibition suppressed the angiogenesis induced by these growth factors (Majima M et al, 1997). Subsequently colorectal cancer cells that over-expressed COX-2 were found to synthesise a number of growth factors including VEGF, bFGF, bFGF-binding protein, TGF-β, PDGF-B, endothelin-1 and inducible nitric oxide synthase (iNOS) and to stimulate both endothelial migration and tube formation. This effect can be inhibited by a variety of angiogenic factor antibodies, selective COX-2 inhibition and by aspirin (Tsuji M et al, 1998). In keeping with these findings is the fact that PGE_2, one of the principal products of cyclooxygenase induced arachidonate metabolism, plays a role in neoangiogenesis and induces the synthesis of angiogenic growth factors such as VEGF (Benefield J et al, 1996)(O'Byrne KJ et al, 2000c). Furthermore COX-2 expression in colorectal and breast cancer cells is associated with activation of MMP-2 and an increase in the expression of MT1-MMPs which both facilitate tumour invasion and angiogenesis (Tsuji M et al, 1997)(Takahashi Y et al, 1999). Finally, COX-2 over-expressing rat epithelial cells show reduced E-cadherin expression coupled with Bcl-2 upregulation and apoptosis resistance This creates a phenotype which in malignant cells favours tumour growth and metastasis (Tsuji M et al, 1995).

Recent data suggests that EGFR activation may result in the upregulation of COX-2 expression (Mestre JR et al, 1997). The induction of COX-2 can be inhibited by both non-specific and specific EGFR tyrosine kinase inhibitors (Sato T et al, 1997)(Yucel-Lindberg T et al, 1999). This demonstrates the importance of tumour-associated cytokines, growth factors and their receptors in the induction of COX-2 activity.

NSAIDs and specific COX-2 antagonists have been shown to inhibit the proliferation and invasive

potential of NSCLC tumour cells *in vitro* (Hida T et al, 1998). They can also enhance the cytotoxicity of conventional chemotherapeutic agents on NSCLC tumour cells (Duffy CP et al, 1998)

Hypoxia and angiogenesis.

Hypoxia frequently occurs in tumours due to the increased oxygen requirement of proliferating cells, increased oncotic tissue pressures and a poor vascular supply with reversible or intermittent flow. Hypoxia is associated with a poor prognosis and resistance to treatment (Vaupel P, 1997). Hypoxia can increase the angiogenic potential of tumour cells by inducing increased expression of VEGF (Schweiki D et al, 1992), bFGF, PDGF (Kuwabara K et al, 1995) and PD-ECGF (Griffiths L et al, 1997). Lung cancers are frequently large and contain necrotic areas. Variations in the extent of intratumoural hypoxia may, at least in part, explain the differences seen in the degree of expression of these angiogenic growth factors by individual cancers.

Hypoxia induces hypoxia-inducible factor-1α (HIF-1α) expression. HIF-1α restores oxygen homeostasis by inducing glycolysis and angiogenesis (Wenger RH et al, 1997). HIF-1α causes growth arrest/apoptosis in a similar manner to p53. Recent work indicates p53 stability is increased by binding HIF-1α (An WG et al, 1998). Furthermore HIF-1α induces p53 and p21 expression while suppressing Bcl-2 expression during hypoxic growth arrest/apoptosis suggests an interaction between these two pathways (Carmeliet P et al, 1998). HIF-1α is a controlling influence on tumour vascularization. Loss of HIF-1α reduces the hypoxia-induced expression of VEGF (Carmeliet P et al, 1998). Interestingly HIF-1α-deficient tumours actually demonstrate increased growth due to decreased apoptosis and 'stress-induced' proliferation (Carmeliet P et al, 1998). EPAS-1 is a recently described endothelial specific hypoxia-inducible protein that has recently been found (Blancher C et al, 1998).

Matrix metalloproteinases and their inhibitors.

The extracellular matrix (ECM) is a framework of proteins and proteoglycans secreted by and surrounding stromal fibroblasts. The ECM gives structural support to cells and plays a central role in cell adhesion, differentiation, proliferation and migration. The turnover of the ECM usually occurs slowly in mature tissues but is accelerated in wound healing, arthritic joint destruction, uterine involution and malignancy (Chambers AF et al, 1997). The first part of the ECM is the basement

membrane. This is made up of type IV collagen and creates a scaffold upon which heparan sulphate, laminin and other components are arranged (Yurchenco PD et al, 1992). All benign epithelial diseases demonstrate a continuous basement membrane (Barsky SH et al, 1983). In contrast a consistent finding in invasive tumours is penetration of the basement membrane (Liotta LA et al, 1983). For penetration of the basement membrane and intravasation to occur there must be alteration of the cell-cell and cell-matrix attachment followed by a proteolytic alteration of the ECM and migration of the tumour cell through the modified matrix. There are several families of enzymes capable of degrading the ECM including the matrix metalloproteinases, serine proteases and cathepsins.

The matrix metalloproteinases (MMPs) are a family of zinc atom dependent endopeptidases (table 1.2). Between them they are capable of digesting all the components of the ECM and the basement membrane. MMPs all contain a catalytic domain that can degrade at least one component of the ECM. They also have a distinctive PRCGVPD sequence in the proenzyme domain that in the latent state has an unpaired cysteine residue that can occupy a co-ordination position on the zinc atom. The disruption of this sulphur-zinc 'bond' activates the enzyme. MMP family members differ structurally from each other by the presence or absence of other domains that affect substrate specificity, inhibitor binding and ECM binding. Most MMPs are secreted as latent proenzymes and require proteolytic activation by the cleavage of an N-terminal sequence in the extracellular space (Birkedal-Hansen H et al, 1993). MMP-11 and MT1-MMP are activated prior to secretion by Golgi-associated furin-like proteases. MT1-MMP has a transmembrane domain and is capable of activating proMMP-2 (He C et al, 1989).

Tissue inhibitors of metalloproteinases (TIMPs) are specific endogenous inhibitors of MMPs which bind to the zinc binding site of active MMPs at molar equivalence (Van Wart H et al, 1990)(Birkedal-Hansen H et al, 1993)(Chambers AF et al, 1997). These inhibitors are secreted by many cells in culture including fibroblasts (Stricklin GP et al, 1983), endothelial cells (Herron GS et al, 1986), chondrocytes (Gavrilovic J et al, 1987) and vascular smooth muscle (DeClerck YA, 1988). ECM turnover is regulated by the balance between activated MMP and free TIMP levels. This balance is important in both physiological and pathological states.

Four TIMPs have been characterised; the core size proteins of these TIMPs are all approximately

Table 1.2 Classification of matrix metalloproteinases and their substrate specificity.

Major subsets	Name	MMP no.	major substrates
CLASSIC MATRIX METALLOPROTEINASE (MMP) MEMBERS:			
COLLAGENASES	interstitial collagenase	MMP-1	fibrillar collagens
	neutrophil collagenase	MMP-8	fibrillar collagens
	collagenase-3	MMP-13	fibrillar collagens
	?	MMP-18	collagens I, II, III, VII denatured collagen
STROMELYSINS	stromelysin-1	MMP-3	laminin, fibronectin, proteoglycans, type IV
	stromelysin-2	MMP-10	collagen
	matrilysin (PUMP-1)	MMP-7	proteoglycans, type IV collagen, gelatins, ECM glycoproteins, elastin
GELATINASES	gelatinase A	MMP-2	type I & IV collagen, gelatin
	gelatinase B	MMP-9	type IV & V collagen, gelatin
ELASTASES	metalloelastase	MMP-12	elastin
	?	MMP-19	elastin
	enamelysin	MMP-20	amelogenin
NOVEL MATRIX METALLOPROEINASE FAMILY MEMBERS:			
RXKR SECRETED TYPE	stromelysin-3	MMP-11	laminin, fibronectin, proteoglycans, alpha 1-antitrypsin
RXKR MEMBRANE TYPE	MT1-MMP	MMP-14	proMMP-2, proMMP-13 fibronectin, collagen I & III
	MT2-MMP	MMP-15	proMMP-2, laminin
	MT3-MMP	MMP-16	proMMP-2
	MT4-MMP	MMP-17	proMMP-2
	MT5-MMP	MMP-21	proMMP-2

adapted from Stetler-Stevenson WG et al, 1996 and Chambers AF et al, 1997

21kDa and contain 12 cysteine residues. The N-terminal is inhibitory whilst the C-terminal has the binding specificity. TIMP-1, -2 and -4 are secreted from cells and remain soluble whereas TIMP-3 binds to the ECM with high affinity (Leco KJ et al, 1994).

TIMP-1 binds with all active MMPs but preferentially with activated MMP-1, MMP-2 and MMP-3 (Apte SS et al, 1995). TIMP-1 also forms a complex with proMMP-9 blocking its activation by stromelysins (Goldberg GI et al, 1992). TIMP-2 preferentially forms a 1:1 complex with MMP-2 and its C-terminal will strongly bind proMMP-2 (Howard EW et al, 1991). At low concentrations, TIMP-2 promotes complex formation with proMMP-2 and MT1-MMP. This formation inactivates MT1-MMP but the proMMP-2 present in the complex can be proteolytically processed to activate MMP-2 by a second molecule of MT1-MMP provided it is free of TIMP-2. Hence low concentrations of TIMP-2 promote MMP-2 activation whilst higher molar ratios of TIMP-2 actually inhibit MMP-2 activation (Strongin AY et al, 1995). TIMP-1, in contrast to TIMP-2, does not inhibit MT1-MMP. TIMP-3 has inhibitory activities against MMP-1, -2, -3 and -9 similar to the action of TIMP-1. TIMP-4 especially binds the C-terminal of MMP-2 and also proMMP-2 (Bigg HF et al, 1997).

Table 1.3 Tissue inhibitors of matrix metalloproteinases.

TIMP	properties		preferred binding site	other binding sites
TIMP-1	29kDa	soluble	proMMP-9, MMP-9	MMP-1, MMP-2, MMP-3
TIMP-2	21kDa	soluble	proMMP-2, MMP-2, MT1-MMP	MMP-1, MMP-3, MMP-9
TIMP-3	27kDa	ECM-bound	MMP-1, MMP-3	MMP-2, MMP-9
TIMP-4	23kDa	soluble	MMP-2	MMP-1, MMP-3, MMP-7, MMP-9

Regulation of MMP expression

MMP expression is normally low but is induced when ECM remodelling is required. Induction usually occurs at the level of transcription, but can also occur due to increased mRNA stability in response to growth factors and cytokines (Johnsen M et al, 1998)(Skobe M et al, 1998). The transcription of most MMPs and TIMPs is regulated by a number of agents including growth factors, cytokines, anti-inflammatory agents, hormones, oncogene products and tumour promoters (Khokha R et al, 1989)(Mann JS et al, 1991)(Leco KJ et al, 1994)(Benbow U et al, 1997). MMP transcription is induced by pro-angiogenic factors such as interleukin-1β (IL-1β), platelet-derived endothelial cell

growth factor (PD-ECGF), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF) and tumour necrosis factor-alpha (TNFα) and is suppressed by transforming growth factor-beta (TGF-β) (Birkedal-Hansen H et al, 1993). Growth factors may induce transcription in a synergistic manner (Pepper MS et al, 1992b). Specific growth factors and cytokines increase the expression of some but not all matrix metalloproteinases (MMPs). TNFα increases MMP-9 and MMP-3 (stromelysin-1) but not MMP-2 (gelatinase A) or MMP-1 (interstitial collagenase) (Hanemaaijer R et al, 1993) whilst bFGF increases production of the gelatinases and interstitial collagenase but not MMP-3 (Mignatti P et al, 1989). In turn MMPs are able to degrade and inactivate IL-1β (Ito A et al, 1996) therefore providing a negative feed-back on MMP transcription. MMPs may cleave the precursor form of TNFα leading to its activation (Moss ML et al, 1997) whilst TIMP-3 inhibits TNFα activation (Amour A et al, 1998). Regulation of MMP activity is also dependent on the activation of their secreted latent proenzymes.

Growth factors may act on tyrosine kinase-specific receptors located in the plasma membrane at the cell surface. Ligand binding will activate the intrinsic kinase activity of the receptor leading to phosphorylation of various cellular substrates. These substrates in turn activate diverse protein kinases and secondary effector molecules. Phosphorylation of these effector substrates, together with alterations in the ionic content of the cell, provide the nuclear signal that activates the transcription of early genes (e.g. *fos* and *jun*) that code for proteins capable of interacting with the regulatory elements of target genes to modulate changes in gene expression (figure 1.4).

The *ras* proteins are members of a superfamily of proteins that in the active state bind to GTP and in the inactive state bind to guanylyl diphosphate (GDP). Most *ras* mutations are defective in GTPase activity and thus are locked into the growth stimulatory GTP-bound form. The *ras* protein signals through a cascade of kinases resulting in the activation of mitogen-activated protein kinases (MAPKs) and the activation of transcription factors including ETS-1 and ETS-2 (O'Hagan RC et al, 1996). This in turn leads to the up-regulation of inducible MMP transcription. *Ras* oncogene expression has been demonstrated to up-regulate MMP-1, -3, -7 and -9 expression in cell culture (Ballin M et al, 1988)(Collier IE et al, 1988)(Meade-Tollin LC et al, 1998) and down-regulates TIMP production (Meade-Tollin LC et al, 1998).

MAPKs are a family of serine/threonine kinases that mediate signals from cell membrane receptors triggered by growth factors, cytokines, hormones and cell-cell and cell-matrix interactions (Lewis TS

Figure 1.4 Regulation of matrix metalloproteinases.
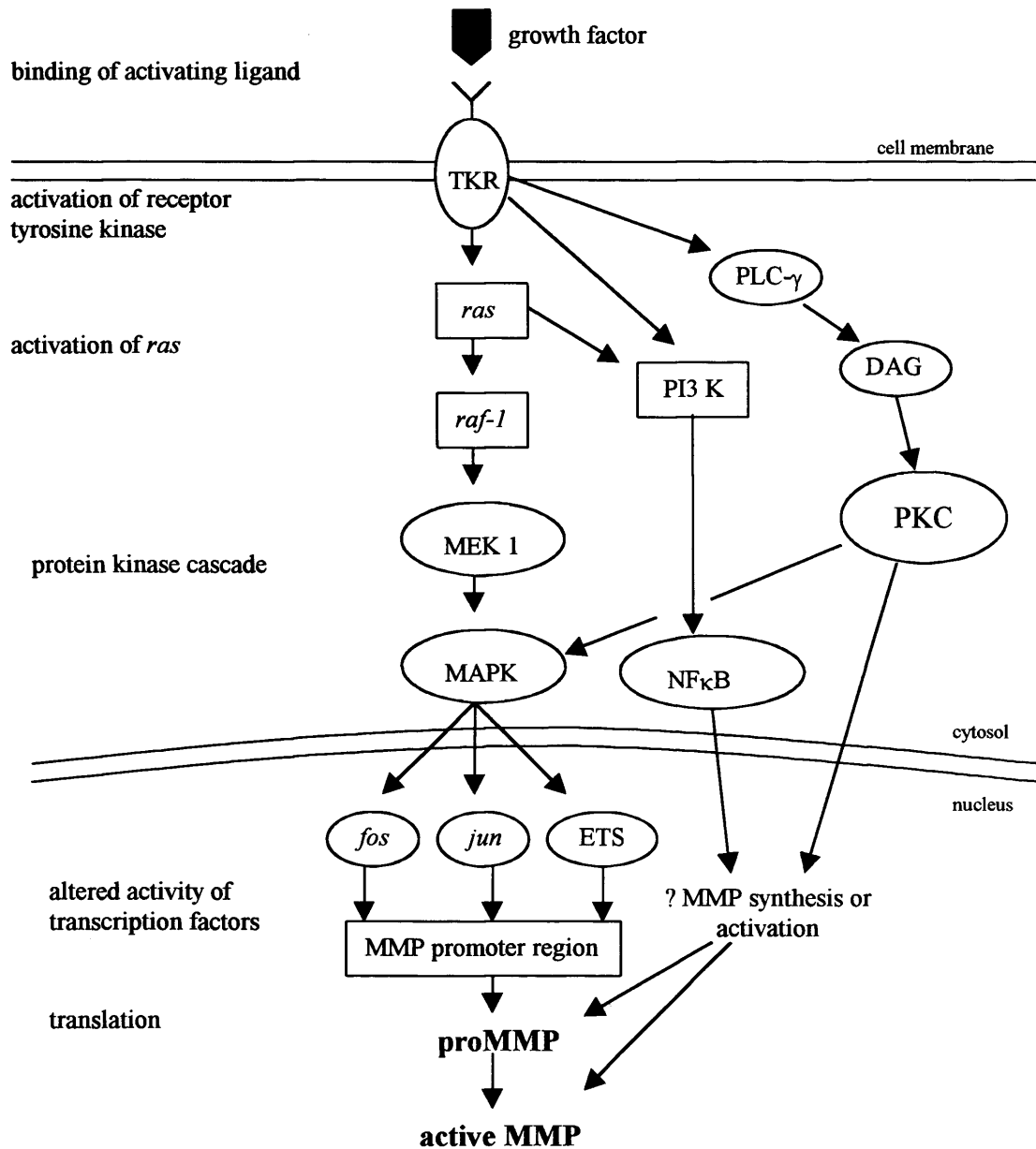
Key: TKR (tyrosine kinase receptor), *ras* (GTP binding protein p21 *ras*), *raf*-1 (a serine threonine kinase activated by *ras*), MEK 1 (mitogen-activated protein kinase kinase 1), MAPK (p42/44 mitogen-activated protein kinase), PI3 K (phosphoinositol-3' kinase), NFκB (nuclear factor kappa B), PLC-γ (γ–isoform of phospholipase C), DAG (diacylglycerol) and PKC (protein kinase C).

et al, 1998). MAPKs regulate the DNA binding and trans-activation capacity of transcription factors. There are three MAPK pathways. The ERK1,2 (ras>raf>MEK1,2>ERK1,2) pathway is activated by mitogens and phorbol esters. The JNK/SAPK (MEKK1-4>MKK4/MKK7>JNK/SAPKs) and p38 (TAK1>MKK3/MKK6>p38s) pathways are stimulated by environmental stress and inflammatory cytokines (Westermarck J et al, 1999). Blocking p38 activity blocks phorbol-ester-induced MMP-9 expression in squamous cell carcinoma and inhibits Matrigel invasion (Simon C et al, 1998) showing cross-signalling by stress and mitogen induced pathways. The relative balance between the activity of these pathways regulates the growth, differentiation and survival of a cell.

Receptor tyrosine kinases also induce the activation of phosphatidylinositol 3' kinase (PI3 kinase) either directly or indirectly via *ras*. PI3 kinase, like *ras*, appears to be an important cell signalling protein involved in several processes, including growth and cell survival (Leevers SJ et al, 1999). PI3 kinase and *ras* inhibitors prevent EGF-induced proliferation and partly reduce EGF-induced MMP-9 secretion in breast epithelial cells (Reddy KB et al, 1999). PI3 kinase can activate nuclear factor (NF)κB which may upregulate COX-2 expression leading to activation of MMP-2 and an increase in the expression of MT1-MMP (Tsuji M et al, 1997)(Lo CJ et al, 1998)(Takahashi Y et al, 1999). There are many other signalling pathways activated by receptor tyrosine kinases including members of the protein kinase C family (PKCs) and the adhesion molecule E-cadherin (Porter AC et al, 1998). Their role in MMP regulation is not fully understood.

The promoter regions of the inducible MMP genes (MMP-1, -3, -7, -9, -10, -12, and -13) show conservation of their *cis* regulatory elements (Benbow U et al, 1997). A single AP-1 element is present in the promoter region of each inducible MMP gene. AP-1 transcription factors are include the *jun* and *fos* families. So far three distinct members of the *jun* family (c-*jun*, *jun* B and *jun* D) and four members of the *fos* family (c-*fos*, Fra-1, Fra-2 and *fos* B) have been characterised (Karin M et al, 1997). Together they form homo and heterodimers with different affinities to the AP-1 site. Decreased expression of the c-*jun*, *jun* B and c-*fos* genes have been observed in human lung carcinomas (Levin WJ et al, 1995).

All inducible-MMP promoter regions (except MMP-12) have highly conserved PEA3 elements adjacent to AP-1 element capable of binding ETS transcription factors. ETS proteins usually form complexes with other transcription factors including AP-1 and can function as co-activators (Carrere

S et al, 1998). Over-expression of ETS-1, ETS-2 and PEA3 enhances MMP-promoter activity (Gum R et al, 1996). ETS-1 expression has been shown to co-localise with MMPs in stromal fibroblasts adjacent to the invasive edge of tumours (Westnert N et al, 1994)(Bolon I et al, 1996). ETS-1 expression has been demonstrated in angiogenesis (Westnert N et al, 1992).

MMPs in invasion, migration and metastasis

Successful tumour invasion necessitates three important steps. The attachment of a cell to the ECM must be weakened whilst a proteolytic defect is formed in the ECM. The detached cell now has to migrate through this defect. Malignant cells must either have sufficient proteinase activity to break through collagenous protein barriers or proteinase activity from host stromal cells. They may also induce proteinase synthesis and release from tumour associated immune cells. MMPs are the only known family of enzymes that can denature and digest fibrillar collagens. As the metastatic cascade requires modification of the matrix it is proposed that MMPs, cathepsins and serine proteases play a significant role. The complex nature of the ECM means that remodelling will require the combined activity of several enzymes. The co-expression of several MMPs may enhance ECM degradation and therefore increase the metastatic potential of a tumour.

There is considerable evidence to suggest that MMPs are actively involved in the metastatic process. Tumour cells, fibroblasts and inflammatory cells all produce ECM-degrading proteases and *in vivo* expression of MMPs is localised to both tumour and stromal cells at the invading margin, providing a mechanism of highly concerted ECM degradation (Johnsen M et al, 1998). MMP concentrations relate to metastatic potential *in vitro* (Garbisa S et al, 1987). Synthetic MMP inhibitors like batimastat decrease the spread of cancer *in vivo* (Talbot DC et al, 1996). Transfection with cDNA for MMP-2, -9 and MT1-MMP also increases metastasis in various cancer cell lines (Sato H et al, 1994)(Kawamata H et al, 1995). Over-expression of TIMP-1 and -2 leads to decreased angiogenesis, endothelial cell migration and invasion (Schultz RM et al, 1988)(Montgomery AM et al, 1994)(Valente P et al, 1998) and antisense down regulation of TIMP-1 mRNA increases invasion (Khokha R et al, 1989).

High levels of MMP expression are found in many malignant tissue types including breast (Basset P et al, 1990), ovarian (Sato H et al, 1992), prostatic (Boag AH et al, 1994), gastric (Nomura H et al, 1995), colonic (Tomita T et al, 1996), thyroid (Sato H et al, 1992) and squamous cell carcinomas of

the head and neck (Muller D et al, 1991) as well as NSCLC (Muller D et al, 1991)(Urbanski SJ et al, 1992)(Brown PD et al, 1993a)(Tokaraku M et al, 1995)(Bolon I et al, 1997)(Kawano N et al, 1997). No single MMP is over-expressed in all tumour cells nor consistently over-expressed in every tumour of a single histological classification. The number of MMP family members detected tends to increase with increased tumour stage and the levels of any individual MMP tends to be higher the more advanced the tumour stage.

Stromal expression of MMPs predominating over the cancer cell expression is a pattern seen with many types of cancer (Basset P et al, 1990)(Polette M et al, 1994)(Nomura H et al, 1995). *In situ* hybridization studies show higher levels of stromal MMPs close to tumour cells with lower levels further away (Pyke C et al, 1993)(Heppner KJ et al, 1996). This suggests the presence of tumour cell-derived factors that are diffusible. EMMPRIN is a tumour-derived factor that increases the expression of several MMPs from stromal cells, in particular MMP-1, -2 and -3 from fibroblasts (Guo H et al, 1997). The stroma also appears to have an important pro-active role in cancer, possibly as a host-response to the tumour. Studies have shown that stromal fibroblasts can cause epithelial cells to become tumourigenic (Atula S et al, 1997)(Skobe M et al, 1998).

Increasing levels of MMP-2 are found as lesions progress from dysplasia through carcinoma *in situ* to frankly invasive carcinoma of breast and prostate (Liotta LA et al, 1983)(Monteagudo C et al, 1990). Increasing levels of MMP and TIMP expression have been described as lesions progress from squamous atypia through to squamous cell lung cancer (Karameris A et al, 1997) supporting a role in NSCLC tumourogenesis. The ratio of active MMP-2 to latent proMMP-2 increases as the tumour grade increases in breast cancer (Davies B et al, 1993a). The activation rate of proMMP-2 is significantly higher in advanced breast cancer with lymph node spread (Iwata H et al, 1996). The presence of proMMP-2 may therefore mark the malignant potential of a cell.

Activation of MMP-2 involves binding of a proMMP-2/TIMP-2 complex to a cell surface receptor followed by cleavage by MT1-MMP. This restricts MMP-2 activity to the pericellular region i.e. the invasive edge of a tumour (Sato H et al, 1994). The integrin $\alpha_v\beta_3$ is capable of binding MMP-2 (Brooks PC et al, 1996). $\alpha_v\beta_3$ is involved in the induction of MMP-2 and TIMP-2 expression in melanoma cell lines (Bafetti LM et al, 1998). This allows MMP-2 activity to be directed towards the pericellular space around invasive cells (Brooks PC et al, 1996) thereby facilitating tumour cell migration and invasion.

30

MMP-9 expression is strongly associated with the metastatic ability of rat embryo fibroblasts; over-expression leading to increased metastatic potential after injection into nude mice (Bernhard EJ et al, 1994)(Hua J et al, 1996). Increasing immunoreactivity for MMP-9 has been shown as bronchial squamous preneoplastic lesions progress from basal cell hyperplasia through metaplasia, dysplasia and carcinoma *in situ* to invasive carcinoma (Galateau-Salle FB et al, 2000). These findings suggest an important role for MMP-9 in the remodelling of the basement membrane and tumourigenesis.

MMP-7 differs from the other MMPs because its expression is in epithelial rather than mesenchymal-derived cells. MMP-7 has been found in both benign and malignant stages of many adenocarcinomas with increased levels in the malignant tissue (Newell KJ et al, 1994)(Heppner KJ et al, 1996). Enhancement of MMP-7 expression in tumour cell lines increases invasive potential and tumorigenesis when inoculated into immunosuppressed host animals (Powell WC et al, 1993) and there is a significant reduction in the number and size of intestinal adenomas in MMP-7-null mice compared with controls (Wilson CL et al, 1997). This may suggest that MMP-7 is responsible for the aggressive behaviour of tumours especially in the early stage.

A higher MMP:TIMP ratio has been found to correlate with the aggressiveness of various solid tumours. Low TIMP expression correlates with increased invasiveness in various murine and human cell lines (Khokha R et al, 1989)(Testa JE, 1992). Rat embryo fibroblasts transfected to over-express TIMP-2 demonstrate a reduced *in vivo* growth rate and decreased local invasion after subcutaneous injection into nude mice. They also show decreased lung colonisation when injected intravenously (De Clerck YA et al, 1992)(Montgomery AM et al, 1994). Spontaneous loss of the metastatic phenotype in a human epidermoid carcinoma line is accompanied by an increase in TIMP-2 expression (Testa JE, 1992). Not all malignancies demonstrate raised MMP activity with decreased TIMP activity. In several reports raised levels of TIMPs have been demonstrated in malignant tissue (Muller D et al, 1991)(Hewitt RE et al, 1991)(Grignon DJ et al, 1996). TIMP-1 expression is associated with the progression of colorectal tumours from adenoma to invasive adenocarcinoma (Muller D et al, 1991)(Hewitt RE et al, 1991). TIMPs may therefore be promoting tumour growth (Hayakawa T et al, 1994). Alternatively over-expression of TIMPs may be an attempt to correct an MMP/TIMP imbalance caused by over-expression of MMPs.

MMPs and growth

The classical view of MMPs and their involvement in malignancy is their role in intra and extravasation via their proteolytic effect on physical barriers. Mounting evidence demonstrates that MMPs can induce growth and tumorigenicity in various cell types. Transfection of cDNA for MMP-7 into colonic carcinoma cells increases *in vivo* tumorigenicity with little change on *in vitro* invasion (Witty JP et al, 1994). A reduction of TIMP-1 can increase tumorigenicity and metastasis in murine 3T3 cells (Khokha R et al, 1989) whilst transfection of TIMP-2 cDNA into melanoma cells decreases both growth and metastasis (Montgomery AM et al, 1994).

In vivo observation of early metastasis by intravital videomicroscopy suggests that the rate-limiting step of metastasis is the growth of newly extravasated cells. The ability of breast cancer cells of low and high metastatic potential to extravasate has been shown to be equal (Morris VL et al, 1994). Overexpression of TIMP-1 showed no effect on extravasation but did decrease the rate of growth after extravasation (Koop S et al, 1994). These cells also demonstrate poor adhesive contacts to vessels and between themselves. MMPs may contribute to the initiation of growth at both the primary and secondary site by altering the local environment e.g. by allowing the access or increasing the release of growth factors into or from the ECM.

Over expression of TIMP-1, -2 and -4 can inhibit tumour growth *in vitro* and *in vivo* which would be expected from their ability to inhibit MMPs (Khokha R et al, 1992)(Imren S et al, 1996)(Wang M et al, 1997). TIMP-1 and -2 can also stimulate growth and proliferation in a variety of cells (Stetler-Stevenson WG et al, 1989)(Bertaux B et al, 1991)(Hayakawa T et al, 1994). TIMP-1 potentiates erythroid tissue (Docherty AJP et al, 1985) and increases fibroblast collagenase production (Clark IM et al, 1994). The growth stimulating effects of TIMPs may be cell-type specific reliant on an appropriate receptor for the TIMP-growth factor domain or dependent on the respective MMP:TIMP concentrations.

MMPs and apoptosis

Apoptosis (programmed cell death) is suppressed in the presence of an intact ECM basement membrane (Boudreau N et al, 1995). Increased MMP-3 expression leads to apoptosis in mammary epithelial cells and this can be blocked by MMP inhibition (Boudreau N et al, 1995). MMPs may therefore be involved in apoptosis by their ability to degrade the ECM. p53 is a promoter of

32

apoptosis, a regulator of the cell cycle and a tumour suppressor gene. The promoter region for the gene encoding MMP-2 contains a p53 binding site. Wild-type p53, but not mutant p53, can upregulate MMP-2 expression *in vitro* (Bian J et al, 1997). This suggests a link between wild-type p53, MMPs and apoptosis. In contrast the mutant p53 frequently found in cancers may have no regulatory control over MMP-2.

Adenovirus-mediated transfer of TIMP-1, -2 and -3 genes leads to over production of their corresponding proteins and to inhibition of melanoma cell invasion (Ahonen M et al, 1998). TIMP-1 and -2 act solely by MMP inhibition, whilst the effect of TIMP-3 is more potent. At 24 hours raised TIMP-3 expression leads to a marked decrease in invasion and cell adhesion. At 72 hours there is an increase in apoptosis and a decrease in viable cells (Ahonen M et al, 1998). Apoptosis was not increased by TIMP-1 or -2 or synthetic MMP inhibition. Therefore TIMP-3 induction of apoptosis is not wholly effected by its ability to inhibit MMPs. An increase in programmed cell death by TIMP-3 also occurs in vascular smooth muscle (Baker AH et al, 1998) and in colon carcinoma cells (Bian J et al, 1996)(Smith MR et al, 1996). Although over expression of TIMP-2 does not lead to apoptosis, it may be asociated with increased necrosis suggesting that it too plays a part in tumour cell survival (Valente P et al, 1998).

MMPs, TIMPs and angiogenesis

The process of angiogenesis has three principal steps which are similar to those involved in tumour invasion: proliferation of endothelial cells, the breakdown of the ECM and endothelial cell migration. There is abundant evidence to show MMPs play an important role in the angiogenic process. MMPs are expressed in the endothelial cells of blood vessels adjacent to tumour cells (Pyke C et al, 1993)(Autio Harmainen H et al, 1993) and MMP-2 can bind to the cell surface of endothelial cells via the integrin $\alpha_v\beta_3$ (Brooks PC et al, 1996). The expression of MMP-2 and -9 in endothelial cells is important in angiogenesis (Johnson MD et al, 1994). MMP-2 leads to endothelial cell tube formation on matrigel (Schnaper HW et al, 1993). Gene targeting has led to the development of MMP-2-deficient mice that develop normally (Itoh T et al, 1997). MMP-2-deficient mice show decreased angiogenesis in their tumours compared to normal mice when injected with B16-BL6 melanoma cells and slower tumour growth and decreased numbers of metastatic colonies in those injected with Lewis lung carcinoma cells (Itoh T et al, 1998). B16-BL6 melanoma cells do not express MMP-2 *in vitro* (Kato Y et al, 1992) so the finding of MMP-2 activity in these tumours in the normal mice but not in

the MMP-2-deficient mice suggests that this MMP-2 has derived from the host tissue. Lewis lung carcinoma cells can produce MMP-2 yet they still demonstrate poorer growth in the MMP-2-deficient mice. Host produced MMP-2 therefore may contribute to tumour invasion and angiogenesis. As there is only partial suppression of angiogenesis in MMP-2-deficient mice this suggests the other proteases partly compensate the lack of this enzyme.

Interstitial and neutrophil collagenases degrade the type-1 collagen that makes up the bulk of the perivascular ECM. Interstitial collagenase (MMP-1) activity is important for angiogenesis *in vitro* (Fisher C et al, 1994) and the expression of MMP-1 may be induced by VEGF at the exclusion of the other MMPs (Unemori EN et al, 1992).

A major event in angiogenesis is stimulation of ECM modelling. bFGF released into the ECM is often bound to heparan sulphate proteoglycans on laminin, fibronectin and collagen (Vlodavsky I et al, 1991b). ECM damage or proteolysis will therefore release bFGF which is proangiogenic. bFGF itself increases gelatinase activity (Pepper MS et al, 1990) and represses TIMP-2 expression (Murphy AN et al, 1993).

MMPs also process precursors into bioactive forms. MMP-2 (but not MMP-9) is capable of cleaving the ectodomain of fibroblast growth factor receptor-1 (FGFR-1) and therefore decreasing the angiogenic potential of bFGF (Levi E et al, 1996). MMPs also proteolytically process membrane-bound TNF-α to yield a secretory active form and can cleave the cell surface TNF recptor to release a soluble ligand-binding receptor fragment (Crowe P et al, 1995). VEGF has been shown to increase MMP-2 expression and decreases TIMP-1 and -2 expression in dermal microvascular cells (Lamoreaux WJ et al, 1998). This finding is supported by a study in NSCLC in which MMP-2 and VEGF expression were closely correlated (Tolnay E et al, 1997). MMP-9 and VEGF co-expression has been demonstrated in NSCLC (Takigawa M et al, 1990) again supporting a close link between MMP expression and angiogenesis.

MMPs are traditionally thought to be proangiogenic whilst TIMPs are antagonistic. There is evidence that the plasmin cascade and MMP activity may also have an antiangiogenic component. MMP-3, -7, -9 and -12 hydrolyse plasminogen to angiostatin, a potent inhibitor of angiogenesis (Dong Z et al, 1996)(Patterson BC et al, 1997). Endostatin is a similar anti-angiogenic substance formed by collagen type XVIII proteolysis (O'Reilly MS et al, 1997). Kringle 5 of human plasminogen also

selectively inhibits endothelial migration (Ji WR et al, 1998). These anti-angiogenic breakdown products may serve as a negative feedback loop. Angiostatin can be produced by the primary tumour and must pass through the circulation as it can be isolated from urine (O'Reilly MS et al, 1994). Excision of the primary tumour can remove this inhibitory effect and lead to rapid growth of previously dormant metastases which is a well recognised observation.

The fumagillin-analogue TNP-470 inhibits the proliferation of growth factor-stimulated endothelial cells and inhibits bFGF-induced tubule formation (Kusaka M et al, 1994). Lung metastases in mice remain dormant under suppression of angiogenesis by TNP-470. Removal of this suppression leads to rapid growth of the micrometastases (Holmgren L et al, 1995). Interestingly whilst the rate of proliferation of cells is equal in dormant and growing metastases, the rate of apoptosis in dormant metastases is three times that of growing metastases (Holmgren L et al, 1995). This suggests that inhibitors of angiogenesis may control the growth of metastases by starving the tumour of nutrients and oxygen and thereby inducing apoptosis.

TIMPs are generally inhibitors of angiogenesis. Although TIMP-1 promotes the proliferation of endothelial cells (Takigawa M et al, 1990), both TIMP-1 and -2 decrease their migration (Johnson MD et al, 1994)(Murphy AN et al, 1993) and inhibit capillary tubule formation *in vitro* (Fisher C et al, 1994). Both TIMP-1 and -2 block the endothelial cell tube formation induced by MMP-2 (Schnaper HW et al, 1993). The maximal expression of TIMP-3 in colorectal adenocarcinomas occurs in the stroma associated with the invasive edge of moderately and poorly differentiated tumours (Powe DG et al, 1997). This pattern of expression is interesting as TIMP-3 has been shown to be antiangiogenic (Anand-Apte B et al, 1997). The effects of TIMPs on angiogenesis can be duplicated using the synthetic metalloproteinase inhibitor batimastat (Fisher C et al, 1994). TIMP-2 and batimastat both inhibit bFGF-induced capillary endothelial cell proliferation (Murphy AN et al, 1993).

Paradoxically TIMP-1 expression is associated with a poor prognosis in NSCLC (Fong KM et al, 1996). Likewise TIMP-2 immunoexpression has been found to correlate with shorter survival in bladder carcinoma (Kanayama H et al, 1998). These findings suggest that TIMPs may actively lead to poor prognosis. Alternatively their expression may be in response increased MMP expression.

Most studies in NSCLC have involved the estimation of mRNA expression by Northern blot analysis, or protein activity by zymography of tumour tissue homogenates. Immunohistochemistry on large patient series is rare. The gelatinases are the most thoroughly investigated MMPs in NSCLC.

MMP-2 expression and activity has been demonstrated in most NSCLC cell-lines (Collier IE et al, 1988)(Suzuki M et al, 1999)(Gonzalez-Avila G et al, 1999). Experiments on human lung carcinoma cell lines show an infiltrating capacity that correlates with a high expression of MMP-2 mRNA (Collier IE et al, 1988).

MMP-2 expression has been demonstrated in tumour, stromal and inflammatory cells in resected NSCLC (Urbanski SJ et al, 1992)(Ohori P et al, 1992)(Brown PD et al, 1993a)(Soini Y et al, 1993)(Nagakawa H et al, 1994). No difference in the levels of MMP-2 and the histological subtype has been demonstrated but the level of MMP-2 has been associated with the invasive behaviour and metastatic potential of most histological types of lung neoplasm (Brown PD et al, 1993a). EMMPRIN, an upregulator of MMP-1, -2 and -3, has been immunolocalised to the surface of NSCLC tumour epithelium and peritumoral stromal cells with MMP-2 expression in the same peritumoural cells (Caudroy S et al, 1999).

No association between MMP-2 and MT1-MMP in normal lung parenchyma has been shown (Nawrocki B et al, 1997). Both MMP-2 and MT1-MMP mRNAs are expressed by stromal fibroblasts of adenocarcinoma of the lung and in the stroma and tumour cells of squamous cell lung carcinomas (Collier IE et al, 1988)(Polette M et al, 1996). Latent MMP-2 binds to the cell surface of malignant cells by interaction with MT1-MMP (Sato H et al, 1996). The expression of MMP-2 in NSCLC can frequently be super-imposed on to that of MT1-MMP (Urbanski SJ et al, 1992)(Tokaraku M et al, 1995). The transcription of these two genes may be co-ordinated leading to MT1-MMP activation of proMMP-2. Other membrane-type MMPs are also capable of activating proMMP-2, but their expression in NSCLC has not been reported on. MMP-2 immunoexpression has been demonstrated in stromal cells closest to cancer cells but also some tumour cells in the peripheral regions of cancer colonies (Nawrocki B et al, 1997).

MMP-9 expression and activity has been found in only a minority of NSCLC-lines (Zucker S et al,

1992)(Suzuki M et al, 1999)(Gonzalez-Avila G et al, 1999) whilst MMP-9 immunoexpression is frequently seen in NSCLC tumour, stromal and inflammatory cells (Muller D et al, 1991)(Brown PD et al, 1993b)(Nagakawa H et al, 1994)(Fujise N et al, 2000). MMP-9 mRNA is present in the stroma closely surrounding tumour cell clusters (Collier IE et al, 1988) and in the tumour cells of squamous cell carcinoma (Cannette-Soler R et al, 1994) and adenocarcinoma (Urbanski SJ et al, 1992). The presence of stroma may therefore be important in inducing tumour cell MMP-9 expression. Elevated levels of circulating plasma MMP-9 occur in patients with NSCLC but have not been shown to correlate with tumour immunoexpression (Iizasa T et al, 1999). No relationship has been shown between MMP-9 and *ras* immunoexpression in NSCLC specimens (Thomas P et al, 2000).

MMP-7 is expressed mainly in the cytoplasm of NSCLC tumour cells but also in fibroblasts (Bolon I et al, 1997). High levels of MMP-7 mRNA are found in squamous and adenocarcinoma whilst low levels are found in non-neoplastic lung tissue (Muller D et al, 1991). One report favours expression in NSCLC adenocarcinoma over squamous cell (Kawano N et al, 1997). MMP-7 positive tumour cells are more common in the peripheral areas of the tumour than in the centre again suggesting a role in tumour cell invasion.

MMP-1, -3 and -11 expression has also been identified in NSCLC (Urbanski SJ et al, 1992)(Bolon I et al, 1997). MMP-1 is found mainly in the stromal cells of lung carcinomas (Bolon I, et al, 1996). MMP-3 transcripts are detected especially in squamous cell lung carcinoma again more commonly in fibroblasts (Urbanski SJ et al, 1992). MMP-11 RNA levels are higher in NSCLC tumours compared to normal tissue (Delebecq TJ et al, 2000). MMP-11 is expressed in bronchial dysplasia and in carcinoma *in situ*, both of which have an increased likelihood of developing invasive squamous cell carcinoma (Bolon I et al, 1996). Increased MMP-11 levels are found in frank squamous cell carcinoma compared with atypia (Karameris A et al, 1997). This suggests that induction of MMP-11 may be an early event in malignant transformation. MMP-11 and uPA are frequently co-expressed (Bolon I et al, 1997) suggesting co-operation in ECM breakdown with MMP-11 degrading α_1 antitrypsin allowing uPA and the other serum proteases to degrade the matrix.

TIMP-1, -2 and -3 are present in both tumour and non-neoplastic lung (Urbanski SJ et al, 1992)(Nagakawa H et al, 1994). TIMP-2 mRNA expression has been shown to increase as the squamous cell passes from atypia through to frank carcinoma (Karameris A et al, 1997). The frequency of TIMP-1 and -2 expression is significantly higher in non-neoplastic lung whereas TIMP-

3 expression tends to increase with malignancy (Nawrocki B et al, 1997). TIMP-1 and -2 mRNA is found mainly in the stroma surrounding tumour cells especially in well-differentiated tumours (Nawrocki B et al, 1997). There is a tendency for TIMP-2 to be immunoexpressed in earlier stage NSCLC tumours (Suzuki M et al, 1999) but TIMP-2 secretion has not shown an inverse relationship with invasiveness in NSCLC cell-lines (Zucker S et al, 1992). TIMP-3 occurs primarily in stromal cells and occasionally in adenocarcinoma cells.

MMPs and prognosis in non-small cell lung cancer

Stage III NSCLC demonstrated significantly higher mRNA levels of both MMP-2 and MMP-9 compared to stage I and II disease (Brown PD et al, 1993a)(Kodate M et al, 1997). A similar association occurs in other tumour types including breast (Allam HS et al, 1993) and colon cancers (Tomita T et al, 1996). Over-expression of MMP-2 is associated with advanced stage (Thomas P et al, 2000) whilst immunoexpression of either MMP-2 and/or MMP-9 has been shown to confer a poor prognosis (Kodate M et al, 1997). This is contradicted by a study of lung adenocarcinomas in which MMP-9 expression was not found to be prognostic (Fujise N et al, 2000). MT1-MMP is expressed more frequently in stage III NSCLC than in stages I and II and increased MT1-MMP mRNA correlates with the presence of lymph node disease (Tokaraku M et al, 1995). Increased levels of MMP-11 RNA have been associated with lymph node spread (Delebecq TJ et al, 2000). Higher numbers of MMP-11 positive fibroblasts and the co-expression of MMP-3, -11 and uPA correlate with increased tumour size and with lymph node spread (Bolon I et al, 1997).

TIMP-3 is more frequently expressed in stage III NSCLC along with many MMPs (Nawrocki B et al, 1997)(Powe DG et al, 1997) whereas TIMP-1 and 2 show a non-significant decline as the tumour stage increases (Nawrocki B et al, 1997)(Suzuki M et al, 1999). The decrease in TIMP-1 and -2 as the stage progresses gives support to the theory that the metastatic potential is dependent on the balance between MMP and TIMP activity. This is at odds with other studies in NSCLC that show TIMP-1 over-expression in advanced disease (Thomas P et al, 2000), high TIMP-1 mRNA levels associated with a poor prognosis (Fong KM et al, 1996) and increased MMP-11 and TIMP-2 expression occurring in poorly differentiated squamous cell carcinoma and in those with nodal metastases (Karameris A et al, 1997). Higher serum TIMP-1 levels also indicate poor prognosis in NSCLC (Ylisirnio S et al, 2000). In both breast and bladder cancer, raised TIMP-1 mRNA and TIMP-2 protein are associated with poor prognosis (Grignon DJ et al, 1996)(Ree AH et al, 1997). There is raised TIMP-1 expression in colorectal cancers with secondary spread (Zeng ZS et al, 1995)

and TIMP-1 expression correlates with a more aggressive phenotype in bladder cancer (Davies B et al, 1993b). Raised levels of TIMPs in malignancy may occur as a response to raised levels of MMPs.

Implications for the treatment of non-small cell lung cancer.

Antiangiogenesis therapy

Inhibiting angiogenesis offers a potential novel therapeutic approach to slowing or reversing tumour growth. The demonstration that angiogenesis occurs in preneoplastic lesions suggests antiangiogenesis strategies could also be effective in smokers at high risk of developing lung cancer (Gazdar AF et al, 2000). There is evidence to suggest a raised microvessel count is associated with a decreased response to chemotherapy (Gasparini G et al, 1995)(Gasparini G et al, 1996). Angiogenesis appears to be very sensitive to small reductions in the factors inducing angiogenesis (Cheng SY et al, 1996). Possible therapeutic avenues include the use of COX-2 inhibitors (Molina MA et al, 1999)(Tomozawa S et al, 1999), retinoids (Lotan R, 1991), fumagillin analogues (Yanase T et al, 1993), interferon-α (Wilkins HE et al, 1997), interferon-γ (Gleave ME et al, 1998) and interleukin-8 antibodies (Arenburg DA et al, 1996). Growth factor receptor antagonists, antibodies and antisense RNA are currently under development (Cheng SY et al, 1996)(Skobe M et al, 1997)(Cardiello F et al, 1999)(Lei W et al, 1999)(Bunn PA et al, 2000). VEGF tyrosine kinase antagonists (Shaheen RM et al, 1999) and EGFR tyrosine kinase antagonists (Vincent PW et al, 2000) appear to be particularly exciting in this regard.

As well as these pharmaceutical agents there are several naturally-occuring angiostatic polypeptides. Angiostatin is a 38kDa fragment of plasminogen produced by proteolysis by macrophage metalloelastase (MMP-12) (Dong Z et al, 1996) stromelysin-1 (MMP-3), matrilysin (MMP-7), gelatinase B (MMP-9) and plasmin (Gately S et al, 1997). Angiostatin is a potent inhibitor of angiogenesis (O'Reilly MS et al, 1994). This peptide has been isolated from primary tumours and accounts for the often seen rapid growth of secondaries after the removal of the primary. After treatment with angiostatin is stopped the tumours grow again but repeated therapy can lead to dormancy without the development of drug resistance (Boehm T et al, 1997). Endostatin is a 20kDa fragment of the C-terminal of collagen XVIII and, like angiostatin, is antiangiogenic *in vitro* and *in vivo*. Endostatin causes the regression of primary tumours whilst the secondaries remain dormant (O'Reilly MS et al, 1997). Intramuscular administration of the endostatin gene leads to biological

expression and inhibition of systemic angiogenesis (Blezinger P et al, 1999).

Antiangiogenic therapy may include the combination of novel antiangiogenic agents with traditional chemotherapy, radiotherapy or surgery. The fumagillin-analogue TNP-470 potentiates the cytotoxicity of cyclophosphamide (Teicher BA et al, 1994). COX-inhibitors enhance the cytotoxicity of conventional chemotherapeutic agents on NSCLC tumour cells (Duffy CP et al, 1998). The ability of angiostatin to cause tumour regression is synergistic with ionising irradiation (Mauceri HJ et al, 1998). Serum VEGF levels increase after thoracic surgery (Maniwa Y et al, 1998) perhaps due to intra-operative hypoxia or as a non-specific response to stress. It has been postulated that raised circulating VEGF levels after surgery coupled with suppression of angiogenesis inhibition may stimulate dormant micrometastases and that peri-operative anti-angiogenesis treatment may be beneficial (Maniwa Y et al, 1998). Antiangiogenesis therapy may require long-term treatment so it will be important that the agents used are well tolerated, have few adverse effects on normal tissue, and allow wound-repair.

MMP inhibition

Synthetic inhibitors of metalloproteinases have been developed and are undergoing clinical trials. Batimastat (BB94) is a hydroxamine analogue which causes widespread inhibition of MMPs. Batimastat decreases *in vitro* ECM degradation and invasion of endothelial cells through an artificial membrane (Brown PD et al, 1995). Batimastat decreases the metastatic spread of murine melanoma (Chiviri RG et al, 1994) and human mammary carcinoma cells in mice (Eccles SA et al, 1996). Malignant effusions contain high concentrations of MMP-2 and -9 (Hurewitz AN et al, 1992) so direct infusion of metalloproteinase inhibitors into the pleural or peritoneal space may offer a new way of approaching this common problem. Batimastat, which has low oral bioavailability, by being placed directly into the pleural or peritoneal cavitybut has been used in clinical trials to treat malignant ascites and pleural effusions (Talbot DC et al, 1996)(Macaulay VM et al, 1999). Batimastat has been shown to significantly reduce the rate of reaccumulation of malignant pleural effusions (Macaulay VM et al, 1999).

Marimastat (BB-2516) is an orally-active synthetic MMP inhibitor that decreases lung and mammary cancer growth in animal models (Wojtowicz-Praga SM et al, 1997). It has been used in phase I, II and III studies. Treatment is associated with a fall in tumour markers in a dose dependent manner

(Rasmussen HS et al, 1997). CT-1746 is a gelatinase-selective MMP inhibitor that reduces tumour spread and metastasis converting aggressive colonic cancer into a more indolent disease in a nude mice model (An Z et al, 1997). CT-1746 has been used in conjunction with cyclophosphamide to inhibit the growth and metastatic spread of Lewis lung carcinoma in mice (Anderson IC et al, 1996). Together they have greater effect than given separately. D-penicillamine can inhibit the activation of prometalloproteinases and offers another way of decreasing MMP activity.

The effects of MMP inhibitors on growth *in vivo* may be related to their effects on tumour angiogenesis. MMP inhibitors block angiogenesis assayed in chick and rat models of neovascularisation (Moses MA et al, 1991)(Taraboletti G et al, 1995). The tetracycline derivative minocycline is an inhibitor of collagenase and leads to decreased angiogenesis *in vivo* (Tamargo RJ et al, 1991).

Long-term treatment with MMP-inhibitors may be used in patients with low or non-existent metastatic disease e.g. in the adjuvant setting. Inhibition of MMP expression by antisense techniques may also have a clinical role. The pharmaceutical use of TIMPs may not be as straightforward as they lack bioavailability and may stimulate cell growth.

Summary.

Further work is required to understand the inter-relationship between the expression of angiogenic growth factors, regulators of apoptosis and expression of proteases in NSCLC. Microvessel counts may be used to identify surgical resection patients who fall into a poor prognostic group and therefore may benefit from adjuvant chemotherapy or radiotherapy. The angiogenic process also offers targets for novel therapies such as MMP inhibitors, receptor tyrosine kinase antagonists and anti-angiogenesis agents, used alone, in combination, and/or in conjunction with, or following, cytoreductive chemotherapy. It is realistic to believe in the near future that the survival from this disease will begin to improve.

Chapter 2

Methods

Methods.

Materials.

Immunohistochemistry.

primary antibodies

CD34: anti-CD34 purified mouse monoclonal antibody (Novocastra Laboratories Ltd). Clone QB-END/10 (isotype IgG_1) recognises human endothelial cells (some cross reactivity with basement membrane collagen) (Martin L et al, 1997a).

EGFR (epidermal growth factor receptor): anti-EGFR purified mouse monoclonal antibody (Novocastra Laboratories Ltd). Clone EGFR.113 (isotype IgG_{2a}) recognises the extracellular region of the EGFR molecule (Fox SB et al, 1994).

c-erbB-2 (neu/HER2): polyclonal antibody HercepTest™ kit (Dako) (Baselga J et al, 1998).

Bcl-2: anti-Bcl-2 mouse monoclonal antibody (Dako). Clone 124 (isotype IgG_{1k}) recognises amino acids 41-54 of human Bcl-2 protein (Pezzella F et al, 1993).

p53: anti-p53 rabbit polyclonal antibody (Novocastra Laboratories Ltd). NCL-p53-CM1 recognises both wild-type and mutant p53 protein (Wolf DC et al, 1995).

MMP-2 (Gelatinase A): anti-MMP-2 mouse monoclonal antibody (Chemicon International Ltd). Clone 42-5D11 (isotype $IgG_{1/k}$) recognises the VTPRDKPMGPLLVATF residue 468-483 of both latent and active MMP-2 (Nomura H et al, 1996).

MMP-9 (Gelatinase B): anti-MMP-9 mouse monoclonal antibody (Chemicon International Ltd). Clone 56-2A4 (isotype $IgG_{1/k}$) recognises residues 626-644 of the carboxy terminal domain of both latent and active MMP-9 (Nomura H et al, 1996).

MT1-MMP: anti-MT1-MMP mouse monoclonal antibody (Chemicon International Ltd). Clone 114-6G6 (isotype $IgG_{1/k}$) recognises residues 160-173 on human MT1-MMP (Nakamura H et al, 1999).

TIMP-1: anti-TIMP-1 mouse monoclonal antibody (Chemicon International Ltd). Clone 147-6D11 (isotype $IgG_{1/k}$) specifically reacts with human TIMP-1 (Fukuda Y et al, 1991).

TIMP-2: anti-TIMP-2 mouse monoclonal antibody (Chemicon International Ltd). Clone 67-4H11 (isotype $IgG_{1/k}$) recognises the carboxy terminal of the TIMP-2 molecule, both native and MMP-bound (Hurskainen T et al, 1996).

secondary antibodies

biotinylated rabbit anti-mouse whole immunoglobulins (Dako)

biotinylated swine anti-rabbit whole immunoglobulins (Dako)

other reagents

protease type 14 (pronase E) (Sigma)

streptavidin–biotin peroxidase complex (ABC) (Dako)

diaminobenzidine tetrahydrochloride (DAB) (Sigma)

3-amino-9-ethylcarbazole (AEC) (Dako)

Epithelial cell culture.

500ml Dulbecco's Modified Eagle Medium (DMEM) (Gibco)

500ml RPMI medium (Gibco)

10% fetal bovine serum (FBS) (Gibco)

25cm^2 and 75cm^2 cell culture flasks (Nalge Nunc International)

500ml sterile phosphate buffered saline (PBS) (Sigma)

0.05% trypsin/0.02% EDTA solution (Gibco)

200nM L-glutamine (Sigma)

dimethyl sulphoxide (DMSO) (Sigma)

epidermal growth factor (EGF) (Sigma)

Reverse transcription-polymerase chain reaction (RT-PCR).

PCR-grade water (Sigma)

0.1% diethylpyrocarbonate (DEPC) (Sigma)

diaminoethane tetra-acetic acid disodium salt (EDTA) (Fisons)

lauryl sulphate (Sigma)

5x AMV RT reaction buffer (Promega)

dNTP mix (Promega)

RnasIn (Promega)

activated charcoal (RA Lamb, Middlesex)

100 base pair ladder (Gibco)

Invasion assay.

growth factor-depleted Matrigel (Becton-Dickinson)

0.1% fibronectin (bovine plasma) (Sigma)

8μm pore polyethylene terephthalate track-etched (PET) membrane (Becton-Dickinson)

Other reagents and chemicals (analar grade).

acetone (CH$_3$COCH$_3$) (Fisher Scientific)

3-aminopropyltriethoxysilane 99% (C$_9$H$_{23}$NO$_3$SI) (Acros Organics)

anhydrous di-sodium hydrogen orthophosphate (Na$_2$HPO$_4$.H$_2$O) (Fisons)

citric acid monohydrate (C$_6$H$_8$O$_7$.H$_2$O) (Fisher Scientific)

DPX mountant (BDH Chemical Ltd)

hydrochloric acid (HCl) (Fisher Scientific)

hydrogen peroxide (H$_2$O$_2$) (Fisher Scientific)

industrial methylated spirit (IMS) 99% (Genta Medical)

methanol (CH$_3$OH) (Fisher scientific)

normal rabbit and swine serum (Gibco)

sodium chloride (NaCl) analytical reagent (Fisher Scientific)

sodium dihydrogen orthophosphate -1 hydrate (NaH$_2$PO$_4$.H$_2$O) (BDH Chemical Ltd)

sodium hydroxide (NaOH) (Fisher scientific)

xylene (Genta Medical)

Immunohistochemistry.

Background.

Immunohistochemistry

Over the last decade immunohistochemistry has emerged as one of the most valuable tools in medical research. This has been primarily due to the increased and widespread availability of monoclonal and polyclonal antibodies, combined with the increasing knowledge of cellular markers. The principle behind the technique is the specific interaction between an antibody and a tissue antigen. This is then labelled and detected using light or electron microscopy. It is imperative that there is good tissue preservation, the tissue antigens are insoluble and their antigenic sites are readily accessible to the antibody.

Antigen Retrieval

There are many methods used to unmask the antigenic sites hidden by hydroxyl-methylene bridge cross-links created during formaldehyde fixation. The most commonly used methods are enzyme digestion or heat-mediated retrieval. Use of enzymes such as trypsin or pronase prior to immunostaining are thought to break cross-linking bonds of the fixative with the protein to reveal the antigenic sites. Over digestion however, may lead to destruction of the tissue matrix. Heat mediated antigen retrieval may also be used. Microwave irradiation of tissue immersed in buffer was first described a decade ago. The problem with this method is that treatment of large batches of slides can produce of 'hot' and 'cold' spots introducing staining inconsistencies. This may be explained by the phenomenon of local superheating as the slides move further away from the point of the microwave focus. Pressure cooking sections briefly in citrate buffer at

130ºC is another heat-mediated method of antigen retrieval. Heating provides the energy to break the hydroxyl bonds formed by the fixative and the protein antigen.

Immunohistochemical methods

There are two immunohistochemical methods. Using the direct method an enzyme or fluorescently labelled antibody binds directly to the antigen. The indirect method has a greater sensitivity because intermediate elements allow for signal amplification. In this project the indirect streptavidin-biotin peroxidase (ABC) method is used. This method is based on four main principles:

1. The affinity between avidin and biotin molecules results in a practically permanent complex.
2. Biotin may be coupled to larger molecules, such as enzymes or antibodies.
3. Avidin can be labelled with a variety of markers such as enzymes, heavy metals and fluorochromes.
4. Avidin can be used as a bridge between two different biotinylated molecules, such as an antibody and peroxidase.

The biotin labelled secondary antibody is applied against the primary antibody. This is then followed by the addition of the avidin-biotin peroxidase complex. Strepatavidin is used instead of avidin, because the former lacks oligosaccharide residues preventing stearic-hindrance and has a neutral isoelectric point, comparative to the neutral pH frequently used in immunostaining. Use of avidin with its higher isoelectric point (10.5) would hence lead to unwanted staining due to reactions with negatively charged tissue components such as cell membranes. Finally a detection system identifies the peroxidase.

The antibodies raised against tissue antigens may be monoclonal or polyclonal. Polyclonal antiserum contains multiple numbers of antibody clones. The problems associated with this type of antiserum are cross-reactivity and non-specific binding. Monoclonal antibodies are populations of antibody molecules derived from a single antibody-producing cell, in which all antibodies are identical and of the same specificity for a given epitope.

Many labelling systems such as fluorochromes, enzymes, colloidal metals e.g. gold and radioactive labels have been used in immunocytochemistry. Peroxidase was the enzyme of choice throughout this study. This binds to hydrogen peroxide and is decomposed to water and atomic oxygen in the presence of an electron donor. On oxidation, these electron donors produce a coloured product. The choice of electron donor was 3-diaminobenzidine

tetrahydrochloride (DAB), which produces a brown end-product that is highly insoluble in water and alcohol.

Study population.

Patients who had undergone a surgical resection for non-small cell lung cancer (NSCLC) in Glenfield Hospital, Leicester between January 1991 and December 1996 inclusive were identified from the histology computer database. Operative nodal sampling or clearance was performed in these patients and this would allow accurate pathological staging of the tumour. Post-operative radiotherapy and chemotherapy were not routinely given. Patient records were reviewed and if necessary the General Practitioner contacted to complete subject follow-up. Follow up for a minimum of 2 years would allow survival analysis to be performed.

Paraffin-embedded formalin-fixed tissue.

Resection tissue was fixed in formalin for 24-72 hours prior to blocks being selected by the histopathologist. Processing of the tissue blocks was carried out in a standard automated fashion at 53-56°C and tissue blocks were then embedded in paraffin wax. Blocks were kept in the dark at 20°C prior to sectioning.

Preparation of cell-line cytoblocks™.

This technique was used to produce paraffin sections of NSCLC cell-lines. Cells grown to 90% confluence were scraped from the base of $75cm^2$ cell culture flasks. Cells were centrifuged at 1000rpm at 4°C for 5 minutes to produce a cell pellet. This pellet was 'fixed' and a cytoblock™ prepared using the cytoblock™ cell preparation system as per protocol (Shandon Inc). This was embedded in paraffin wax for future sectioning.

Tissue sectioning.

All the immunohistochemical techniques were performed on paraffin-embedded tissues. 4μm sections were cut onto glass slides which were previously treated with 2% 3-aminopropylethoxysilane (in methanol). Paraffin sections were cut and dried overnight at 37°C followed by 6 hours at 56°C to assist adhesion of the tissue. Slides were stored in the dark at 4°C until required. Studies showed no deterioration in staining in control sections kept in this manner for 6 months or 1 year. Slides used for the HercepTest™ were cut within six weeks of

immunostaining. Repeat HercepTest™ testing on sections cut twelve months previously showed no obvious loss of antigenicity.

For immunohistochemistry, sections were dewaxed in xylene and re-hydrated through a series of alcohols (2x 99% alcohol, 95% alcohol) with periodic agitation for two minutes in each solvent.

Antigen Retrieval.

Pressure cooking:

The dewaxed slides were rinsed in de-ionised water and immersed into a pressure cooker (Prestige cooker, model 6189) containing 1500ml pre-heated citric acid buffer* (for * see appendices). The lid was locked and the steam regulator was placed at the one dot position. When the large pressure indicator rose, the sections were subjected to 2 minutes treatment. The slides were removed and washed in running tap water for 10 minutes, then rinsed in de-ionised water for 5 minutes.

Microwaving:

The dewaxed sections were rinsed in de-ionised water. Up to 25 slides were subsequently positioned in plastic racks and immersed in 500ml citric acid buffer. The slides were placed in a Proline 800W microwave for 12 minutes. The slides were then removed and washed in running tap water for 10 minutes, then rinsed in de-ionised water for 5 minutes.

Protease digestion:

The dewaxed sections were rinsed in de-ionised water. The slides were placed into a Hellendahl jar containing phosphate buffered saline* (PBS) pH 7.4 at 37°C for 5 minutes. The slides were then placed in a Hellendahl jar containing a solution of 0.1% protease type 14 (pronase E) and 0.01% calcium chloride in PBS pH 7.4 at 37°C for 10 minutes. The slides were removed and washed in running tap water for 10 minutes, then rinsed in distilled water for 5 minutes.

HercepTest™:

Dewaxed sections were rinsed in de-ionised water. The slides were then placed into a Hellendahl jar containing HercepTest™ epitope retrieval solution at 97°C for 40 minutes, then room temperature for 20 minutes. The slides were then placed in HercepTest™ wash buffer for 5 minutes.

Antibody storage.

On arrival, antibodies were split into small working-volume aliquots and kept at -20°C until required. Antibody thawing and refreezing was kept to an absolute minimum. Studies showed that antibodies could be kept in this manner for at least 2 years without affecting staining.

Streptavidin-biotin complex peroxidase technique.

After treatment in tris buffered saline* (TBS) for 5 minutes, slides were placed in a humid chamber on a rocker platform after wiping off excess buffer from around the sections. The sections were covered with 100μl 20% serum at room temperature for 15 minutes to block non-specific staining. The choice of serum was determined by the species with which the secondary antibody had been raised. Excess serum was drained and wiped away and 100μl of the diluted primary antibody in 20% serum was then applied and incubated overnight at 4°C (table 2.1).

At the end of the incubation period, the primary antibody was rinsed off with TBS and then washed in fresh TBS for 5 minutes. The slides were then replaced onto the humid chamber and incubated with 100μl of appropriate secondary antibody for 30 minutes. Immediately after applying the secondary antibody the streptavidin–biotin peroxidase complex (ABC) was prepared as follows:

TBS:	1000μl
streptavidin:	1μl
biotinylated horseradish peroxidase:	1μl

The secondary antibody was rinsed off with TBS and washed in fresh TBS for 5 minutes. The slides were dried around the sections and 100μl of ABC solution applied for 30minutes. The ABC solution was rinsed off with TBS and the slides washed in fresh TBS. Sections were then returned to the humid chamber and incubated with 100μl of diaminobenzidine tetrahydrochloride (DAB) solution* for 10 minutes. Sections were then rinsed briefly in TBS, washed in running tap water and counterstained in Mayers Haematoxylin*. The sections were then dehydrated through graded alcohols (95% alcohol, 2x99% alcohol) and xylene, then mounted in DPX using appropriately sized coverslips.

Preliminary experiments were conducted in order to establish the most effective conditions and reagents. Information from company data sheets was used to obtain approximate primary antibody dilutions. Other dilutions around these values were also tested and compared.

49

Table 2.1 Summary the antibodies and conditions used for immunohistochemistry on formalin-fixed, paraffin-embedded tissue.

	CD34	p53	Bcl-2	EGFR	c-erbB-2	MMP-2	MMP-9	TIMP-2
Antigen retrieval	nil	pressure cooking 2 minutes	microwave cooking 2 minutes	pressure cooking 2 minutes	'epitope retrieval' 97°C 40 minutes	pressure cooking 2 minutes	pressure cooking 2 minutes	pressure cooking 2 minutes
Blocking serum	rabbit	swine	rabbit	rabbit	goat	rabbit	rabbit	rabbit
Primary antibody	Novocastra QB End/10	Novocastra p53-CM1	Dako Clone 124	Novocastra EGFR.113	Dako 'HercepTest'	Chemicon 42-5D11	Chemicon 56-2A4	Chemicon 67-4H11
Dilution	1:50	1:800	1:25	1:20	protocol	1:50	1:100	1:100
Incubation	overnight 4°C	overnight 4°C	overnight 4°C	overnight 4°C	30 minutes room temp.	overnight 4°C	overnight 4°C	overnight 4°C
Secondary antibody	rabbit anti-mouse immunoglobulins	swine anti-rabbit immunoglobulins	rabbit anti-mouse immunoglobulins	rabbit anti-mouse immunoglobulins	rabbit anti-goat immunoglobulins	rabbit anti-mouse immunoglobulins	rabbit anti-mouse immunoglobulins	rabbit anti-mouse immunoglobulins
Dilution	1:400	1:600	1:400	1:400	protocol	1:400	1:400	1:400
Buffer	TBS	TBS	TBS	TBS	'Herceptest wash'	TBS	TBS	TBS

Immunostaining for MT1-MMP (Chemicon 114-6G6) and TIMP-1 (Chemicon 147-6D11) did not elicit evaluable staining.

For each section a negative control was included, where the primary antibody step was omitted and the serum was left on. In addition a positive control preparation tissue, known to contain the antigen in question was carried through with every batch of immunostain to confirm that reagents were in working order and to allow assessment of staining between batches. For many antibodies the tissue also contained an internal positive control.

Evaluation of angiogenesis.

Angiogenesis can be measured indirectly in solid tumours by counting microvessels. Intratumoral microvessels can be identified by immunohistochemical staining of endothelial cells. The first report to associate tumour microvessel counts with metastatic spread was published in 1991 (Weidner N et al, 1991). Their method employed the identification of areas within tumour tissue sections with an elevated vascular density and subsequent counting of the number of microvessel entities within these 'hot spots'. Sections from the periphery of the tumour are used as these are usually the most vascular and there is least necrosis. Based on the hypothesis that the 'hot spot' increases the entrance of surrounding tumour cells into the circulation, only 'hot spots' within a high powered field of viable tumour clusters are included. 'Hot spots' within areas of necrosis are not included.

Weidner described any immunostained endothelial cell/cell cluster separated from adjacent microvessels or tumour/stromal cells as a distinct countable microvessel. A lumen or the presence of red blood cells was not required, nor was a cut-off calibre size. Distinct areas of immunostained endothelial cells that appear to be transected by the plane of the tissue section more than once are counted as separate microvessels. The inter-observer error in vessel counting has been assessed by taking photographs of highly vascular areas. Correlation of vessel counts performed by experienced and inexperienced observers on these photographs was very strong (Vermeulen PB et al, 1997). This suggests that vessel counting after agreement on the 'hot spot' is reliable. However the experience and training of the observer does appear to determine the ability to determine the 'hot spot' (Vermeulen PB et al, 1997). The most important observer-dependent step is therefore the selection of the vascular 'hot spot'.

More recently the Chalkley eye-piece graticule has been used to facilitate the measurement of tumour angiogenesis. This graticule contains 25 randomly positioned dots (figure 3.1) and at high power is orientated so that the maximum number of points on or within the vessels of the vascular 'hot spot' are counted (Fox SB et al, 1995). The Chalkley count should be a more objective approach than microvessel counting.

As well as microvessel counting, a semi-quantitative grading system (low/medium/high) has also been validated. This highly subjective technique requires a long period of training to reduce inter-observer variation. The advantage of a subjective grading system is the reduced time required assessing sections (Fox SB et al, 1995).

In an attempt to increase objectivity, attempts at semi-automated vessel counting using computerised image analysis systems have been assessed. The additional advantage of these systems is that morphometric parameters can be measured, for instance vessel dimensions, total area of 'vasculature' or lumen size (Fox SB et al, 1995). The total section is also more easily counted and the results less dependent on the experience of finding the 'hot spot'. Potentially laser scanning cytometer technology using immunofluorescent antibodies may also be employed.

Figure 2.1 Chalkley graticule.
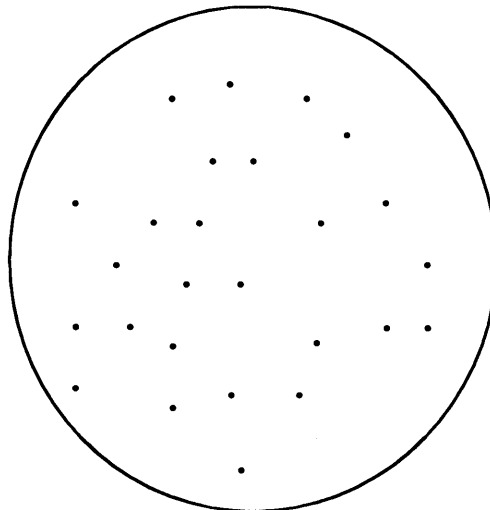
Antibodies can be either pan-endothelial cell markers or markers of activated/proliferating endothelium. Analysis of intratumoral microvessels in breast cancer has suggested that anti-CD34 is a more reliable and reproducible antibody for routine studies (Martin L et al, 1997a)(table 2.2).

In this study, the method used for microvessel assessment was that proposed by an international consensus on the methodology and criteria of evaluation for the quantification of angiogenesis in solid human tumours (Vermeulen PB et al, 1996). I underwent training in 'hot spot' identification, microvessel counting and the use of the Chalkley eye-piece graticule by Russel Leek at the ICRF Molecular Oncology Laboratory, Institute of Molecular Medicine, John Radcliffe Hospital in Oxford. The pan-endothelial antibody to CD34 was chosen for the best balance of reliability, specificity and sensitivity (Martin L et al, 1997a). Staining was performed

on 4μm thick paraffin-embedded formalin-fixed tumour sections from the tumour periphery. Under low power three 'hot spots' were identified. These were then viewed under high power (x250) using a Chalkley eye-piece graticule (Graticule, Surrey) by two investigators to give a Chalkley count.

Table 2.2 Endothelial cell specific antibodies.

antibody	sensitivity	specificity	frozen	reactivity paraffin	lymphatic staining
anti-FVIII-RAg	large vessels capillaries-focal and variable polyclonal Ab more sensitive	monoclonal Ab - high polyclonal Ab- stromal, tumour and inflammatory cell staining requires antigen retrieval	+	+	proportion
anti-CD31	large and small vessels equal intensity	high- occasional plasma/ inflammatory cell staining requires antigen retrieval	+	+	no
anti-CD34	large and small vessels equal intensity	high- variable basement membrane staining reproducibility > CD31	+	+	proportion
UEA	large and small vessels equal intensity	low-tumour cell staining	+	+	yes
PAL-E	large and small vessels equal intensity	high	+	-	no

adapted from Vermeulen PB et al, 1996, Martin L et al, 1997a

The use of computer-image analysis to count vessels was performed using NIH Imaging on an Apple Macintosh G-3 computer. Images were captured using a JVC KYF50 3-chip colour video camera and a Scion cg-f frame grabber. A series of computer macros would potentially allow morphometric measurements of vessel size, length, luminal size and total area to be analysed. This system would be able to provide data for the entire tumour section and to remove the subjective elements of hot spot identification and microvessel counting. Initially the sections stained with DAB and counterstained with Haematoxylin were used. Difficulty separating the brown and blue colours by digital-colour extraction was encountered. A series of studies revealed slides immunolabeled with red-pigmented 3-amino-9-ethylcarbazole (AEC) and a neutral green counterstain to be the most easily interpreted.

Fluorescence in-situ hybridisation.

Pre-treatment and in situ hybridisation for c-erbB-2 was performed using the 'Paraffin pre-treatment reagent kit' (Vysis) and the 'PathVysion DNA probe kit' (Vysis) respectively. Slides

were processed according to the protocols provided with the kits except for the following deviations. Firstly slides were immersed in protease for 20 minutes. Secondly probes and target DNA were not denatured separately but by co-denaturation. The Vysis hybridisation probe mix was applied to the dried slides, coverslipped, sealed with rubber-cement and co-denatured on a hot-plate at 75°C for 6 minutes. Thirdly the post-hybridisation washes were 2x 5 minutes in 50% formamide in 2x standard saline citrate (SSC) pH 7.0-8.0 at 42°C, 2x 5 minutes in 4x SSC with 0.05% Tween-20 pH 7.0-8.0 at room temperature then 5 minutes in 4x SSC pH 7.0-8.0 at room temperature. Slides were rinsed in distilled water and mounted in antifade with DAPI.

Lung cancer cell-lines: source, morphology and culture.

All cells were originally obtained from the American Type Tissue Collection, but for this study were received as gifts from Professor A. Wardlaw, Department of Respiratory Medicine, Glenfield Hospital, Leicester; Dr J. Coulson, CRC laboratory, Nottingham City Hospital and Dr A. Patterson, Department of Pharmacology, University of Manchester. All the cell-lines were confirmed to be mycoplasma-free by the donating laboratory.

NCI-H460 (Chinje EC et al, 1999)
adenocarcinoma
RPMI + 10% FBS
split 1:10 twice weekly

A549 (Chinje EC et al, 1999)
adenocarcinoma
DMEM + 10% FBS
split 1:10 weekly

MOR-P (Rhodes T et al, 1992)
adenocarcinoma
RPMI + 10% FBS
split 1:10 weekly

SK-MES (Favoni RE et al, 1994)
squamous cell carcinoma
DMEM + 10% FBS
split 1:10 weekly

NCI-H647 (Chinje EC et al, 1999)

adenosquamous cell carcinoma

RPMI + 10% FBS

split 1:10 twice weekly

Epithelial cell culture.

General.

Cells are routinely maintained in an incubator (Heraeus) at 37°C in a humidified atmosphere of 5% CO_2/95% air. Cells are manipulated under aseptic conditions in a Class II laminar flow tissue culture cabinet (Gelaire, ICN). To ensure optimal viability of cells, they should be frozen slowly and thawed quickly. Ideally cells should also be in log phase of growth (i.e. sub-confluent) prior to freezing.

Routine culture.

The culture medium was either a) Dulbecco's Modified Eagle Medium (DMEM) with L-glutamine and D-glucose (without sodium pyruvate) and 10% fetal bovine serum (FBS) or b) RPMI with L-glutamine and 10% FBS. The cells were cultured in $25cm^2$ and $75cm^2$ cell culture flasks.

To split confluent cultures, the media was removed and the cells washed with sterile phosphate buffered saline (PBS). 0.05% trypsin/0.02% EDTA solution, 1ml per $25cm^2$, was added and dispersed over the adherent layer by gentle shaking. The flask was returned to the incubator for 1-4 minutes and the cells detached by tapping. Appropriate medium was added and the cells spun at 1000rpm at 4°C for 5 minutes. Excess media was decanted and the cells were resuspended in appropriate media and split 1:10 to allow seeding at 3-6 x 10^4 cells per cm^2.

A bank of cells was created by freezing resuspended cells in freezing medium and placing them in a polystyrene padded box in a –80°C freezer overnight prior to transfer to the gaseous phase of a liquid nitrogen canister. 1ml of freezing media* was added to each set of resuspended cells from a $25cm^2$ flask and placed in a 1.8ml cryotube.

Stimulation with EGF.

$2x10^5$ cells were seeded per well in a 12-well plate in appropriate medium containing 10% FBS and 100ng/ml epidermal growth factor (EGF). After 48 hours the media was removed and frozen at –80°C for future zymographic analysis. mRNA was extracted from the cells as described below.

mRNA extraction with oligo (dT)$_{25}$ Dynabeads*

General precautions.

The critical aspect of any RNA isolation procedure is the protection of the sample from contamination with ribonucleases (RNases). RNases are stable enzymes and do not require co-factors for their action. Therefore even a small amount of RNase in a sample can lead to RNA destruction. The major source of contamination is the gloves of the researcher and these should be changed regularly. All plastic-ware should be autoclaved and dried (80°C for at least 4 hours). Autoclaving will not fully inactivate RNases and water or salt solutions should be treated with 0.1% diethylpyrocarbonate (DEPC), an RNase inhibitor, for at least 1 hour followed by autoclaving to remove traces of DEPC. Tris-based solutions can not be dealt with in this manner as they react with DEPC. Tris should be dissolved in water that has already been treated with DEPC and autoclaved.

Cells.

A cell lysate is produced from a monolayer of cells by washing in sterile cold PBS, application of trypsin to free the cells and centrifugation to yield a cell pellet. The pellet is resuspended in cold PBS and a cell count is performed. This allows extraction from a defined number of cells which enables comparisons of gene expression to be made between samples.

10^5 cells were resuspended in sterile cold PBS and centrifuged to form a pellet. The supernatant was discarded and 250μl of lysis-binding buffer* added. Lysis of the cell suspension was achieved by passing it repeatedly through the pipette tip. If the suspension was particularly viscous, the lysate was forced through a 21G needle to shear DNA strands. The lysate was placed on ice for 30 minutes.

mRNA extraction.

The oligo (dT)$_{25}$ Dynabeads were resuspended and 30μl was transferred to a sterile eppendorf tube. The eppendorf was placed in a Dynal Magnetic Particle Concentrator (MPC)* and when the suspension cleared, the supernatant was removed. The eppendorf was removed from the MPC and the prime beads resuspended with 50μl lysis-binding buffer. The eppendorf was replaced in the MPC and the supernatant removed. The cell lysate was added to the oligo (dT)$_{25}$ Dynabeads and mixed thoroughly with gentle pipetting. To allow annealing between oligo (dT)$_{25}$ Dynabeads and mRNA, the solution was kept at 4°C for 30 minutes. Dynabead-bound mRNA was recaptured by placing the eppendorf in the MPC and leaving for 2 minutes before removing the supernatant. The eppendorf was removed from the MPC and the bead-bound

mRNA washed with buffer + SDS*. The eppendorf was replaced in the MPC and the supernatant removed. This step was repeated once. The bead-bound mRNA was then washed three times with wash buffer* as above. Repeated washing was required to remove any DNA from the samples. Finally the beads were resuspended in 100µl RNase-free PCR grade water. Dynabead-bound mRNA can be stored at −80°C for up to 1 year without degradation.

Reverse Transcription.

Background.
For detection or quantification of mRNA by PCR it is first necessary to reverse transcribe the mRNA into complementary DNA (cDNA) with a reverse transcriptase (RT) reaction. The quality of the cDNA depends on the integrity of the mRNA and the fidelity of the transcription. The cDNA is synthesised from RNA by extension of an annealed primer. These can be random, specific or oligo dT primers. mRNA extracted with oligo dT Dynabeads is already annealed to the oligo dT so no further priming for cDNA synthesis is required.

The typical yield of total RNA from 10^6 cells is between 600 and 1600µg. mRNA represents 1-5% of total RNA therefore the mRNA yield from 10^5 cells will be 0.6-8µg.

Reverse Transcription.
All solutions for RT are stored at −20°C. Pipettes should be dedicated to RT-polymerase chain reaction (PCR) and all equipment should be UV irradiated for 20 minutes before use.

Bead-bound mRNA was placed in the MPC to pellet the beads and then washed three times with 25µl RT buffer*. The beads were resuspended in 20µl PCR grade water and divided into 2 x 10µl aliquots in two 0.5ml eppendorf tubes.

Two master-mix solutions in the following ratio of stock solutions were made up.

	RT +ve	RT −ve
5x AMV RT buffer*	55µl	55µl
dNTP mix (10mM) *	44µl	44µl
RnasIn (40U/µl)*	11µl	11µl
AMV RT (10U/µl)*	8.8µl	-
PCR grade H₂O	46.2µl	55µl
Total volume	165µl	165µl

15µl of the RT+ve mix was added to one 10µl aliquot and 15µl of RT-ve mix added to the other aliquot. This was overlain with sterile mineral oil and incubated at 42°C for 60 minutes.

A control containing 10µl PCR water instead of Dynabeads was included to test for contamination of master-mix by DNA following subsequent PCR.

Polymerase Chain Reaction.

One of the major risks with PCR is contamination by extraneous DNA so PCR should be set up in a dedicated area away from samples of amplified DNA. Dedicated pipettes should be used and gloves changed frequently during the procedure.

PCR set up.

The PCR mastermix (sufficient for 9 reactions) was made up as follows:

10x AJ buffer*	50µl
forward & reverse primers (10pmole/µl)	10µl each
PCR grade H_2O	420µl for GAPDH
	410µl for MMP-2, -9, MT1-MMP, TIMP-1 and -2

49µl of mastermix was added to each eppendorf for GAPDH PCR and 48µl for MMP-2, -9, MT1-MMP, TIMP-1, and -2 PCR. 1µl cDNA was added for GAPDH PCR and 2µl cDNA for MMP-2, -9, MT1-MMP, TIMP-1 and -2 PCR. The mastermix/cDNA mixture was covered with sterile mineral oil to minimise evaporation. 1µl taq polymerase (1U/µl) was added to each eppendorf after the initial denaturation step to minimise non-specific priming during the PCR set up.

PCR Programme.

cycle	denaturation	annealing	extension
1	94°C, 5 minutes	#°C, 1 minute	72°C, 1 minute
n	94°C, 1 minute	#°C, 1 minute	72°C, 1 minute
last	94°C, 1 minute	#°C, 1 minute	72°C, 7 minutes

For each primer set, initial studies were performed using cDNA from the HT1080 fibrosarcoma cell-line to establish the optimal annealing temperature. This was found to be:-

59°C for GAPDH, TIMP-1, TIMP-2

60°C for MMP-2, MT1-MMP

66°C for MMP-9

The PCR program was performed for both 30 and 35 cycles for each primer.

Controls.

For each sample, PCR was performed in parallel on a negative RT sample to control for any genomic DNA or other DNA contamination during the extraction process. In order to allow some comparison of the relative amount of gene expression between samples and also to control for the efficiency of mRNA extraction, PCR was performed on each case using the housekeeping gene GAPDH. In each PCR batch, a positive control (cDNA from HT 1080 cells) and a negative control (PCR water in place of cDNA) was included to control for the reaction process and reagents.

Oligonucleotide primers.

Oligonucleotide primers were designed in-house by Dr Louise Jones and Dr Howard Pringle, using the Molecular Biology Suite 'gcg' primer design software PRIMER. Where possible primers were designed to amplify across exon boundaries. Specificity of the primers to the target gene was checked against the European Molecular Biology Library (EMBL) and GENBANK databases using FINDPATTERNS on the 'gcg' sytstem. The oligonucleotides were synthesised by Gibco BRL, Oswell and Genosys, and the forward primer of each primer set was synthesised biotinylated.

Table 2.3 Oligonucleotide primer sequences.

Gene	primers (5'-3')	Size
GAPDH	**F:** AGA ACA TCA TCC CTG CCT C **R:** GCC AAA TTC GTT TGC ATA CC	350bp
MMP-2	**F:** ATT GAT GCG GTA TAC GAG GC **R:** GGC ACC CTT GAA GAA GTA GC	350bp
MMP-9	**F:** TTC TAC GGG CCA CTA CTG TGC **R:** CGC CCA GAG AAG AAG AAA AG	250bp
MT1-MMP	**F:** TCG CCA ATG GAA AGA CCT AC **R:** TGA TGA TCA CCT CCG TCT CC	315bp
TIMP-1	**F:** TGG GGA CAC CAG AAG TCA AC **R:** CAG GGG ATG GAT AAA CAG GG	350bp
TIMP-2	**F:** AAC GAC ATT TAT GGC AAC CC **R:** ACC TGT GGT TCA GGC TCT TC	250bp

Agarose Gel Electrophoresis.

Amplified cDNA can be identified by agarose gel electrophoresis separating DNA on the basis of size. If the size of the PCR product is known (as predicted by the known cDNA sequence)

then its presence can be documented by staining the gel with ethidium bromide followed by comparison with a ladder of DNA fragments of known size. Ethidium bromide is a mutagen and so gloves must be worn and care taken in its handling and disposal. Diluted solutions of ethidium bromide can be decontaminated by adsorption over activated charcoal. Ethidium bromide is a fluorescent dye that detects both single and double stranded DNA. At 254nm, UV light is absorbed by the DNA and transmitted to the dye. The energy is re-emitted at 590nm in the red-orange region of the visible spectrum. The least amount of DNA in a single band that can be reliably detected with ethidium bromide is approximately 10ng. The most DNA compatible with a sharp band is 100ng. Overloaded DNA results in trailing and smearing, a problem that increases as the size of the DNA increases.

The distance used to determine voltage gradients is the distance between the electrodes, not the gel length. The gel should be run until the band of interest has migrated 40-60% down the length of the gel.

Method.

To prepare 2% agarose gel, 3g agarose powder was added to 150ml Tris/Acetate/EDTA Buffer (TAE)* in a 500ml conical flask, placed in a microwave and set to high for 1 minute. The solution was agitated and then heated and agitated in short bursts until the agarose gel fully dissolved. 2µl of ethidium bromide was added and the solution allowed to cool prior to pouring into a midi gel holder with a comb to a depth of 3-4mm. Air bubbles were removed and the gel allowed to set. The comb was removed and the gel immersed in TAE in an electrophoresis tank.

5µl 5x loading buffer* was added to 20µl cDNA in a sterile eppendorf. A 100 base pair ladder was added to the first well and 20µl of cDNA/loading buffer added to each subsequent well. The gels were run at 90V and at maximum current towards the positive electrode for approximately 45 minutes and the gel photographed in the darkroom.

Invasion assays.

Background.

Invasion of cancer cells through an artificial basement membrane has been used as an effective *in vitro* model of *in vivo* invasiveness (Albini A et al, 1987). As EGF-upregulation of MMP-9 expression may be expected to contribute to an invasive phenotype, this study compared base-line invasiveness with invasion after stimulation with 100ng/ml EGF in 5 NSCLC-lines. This concentration of EGF was chosen on the basis of other work performed in the laboratory on

breast cancer cell-lines and on the published literature demonstrating major effects at this concentration

Invasion assay set-up.

Well inserts supporting an 8μm pore polyethylene terephthalate track-etched (PET) membrane were suitable for culture in a 12 well cell culture plate. Fibronectin (1mg/ml) was diluted 1 in 100 with cold PBS to give a final concentration of 10μg/ml. 200μl of the diluted fibronectin was added to the base of the PET membrane of each well insert for 30 minutes before the excess fibronectin was removed and the membrane allowed to dry for 1 hour.

Growth factor-depleted Matrigel was thawed overnight at 4°C. All plasticware was pre-cooled prior to use and all reagents kept on ice. Matrigel (10mg/ml)* was diluted 1 in 100 with cold serum-free cell medium to give a final concentration of 100μg/ml and 100μl added to the upper surface of each well insert and incubated at 37°C for 2 hours. The excess was removed and the wells washed twice in serum-free cell medium.

A 1:1 mixture of appropriate fibroblast conditioned medium* with serum-free cell medium was made up and 1ml added to each well. 0.5ml serum-free cell medium was added to each insert. 5×10^4 cells, grown to 70-90% sub-confluence, were added to 2 inserts per invasion assay (the number of cells being that to achieve 70-90% sub-confluence in the insert). The cells were incubated under standard conditions for 48 hours (figure 2.2). The addition of 50ng EGF (concentration 100ng/ml) to the serum-free media in the upper well at 2 hours was used for EGF stimulation.

Figure 2.2 Invasion assay.
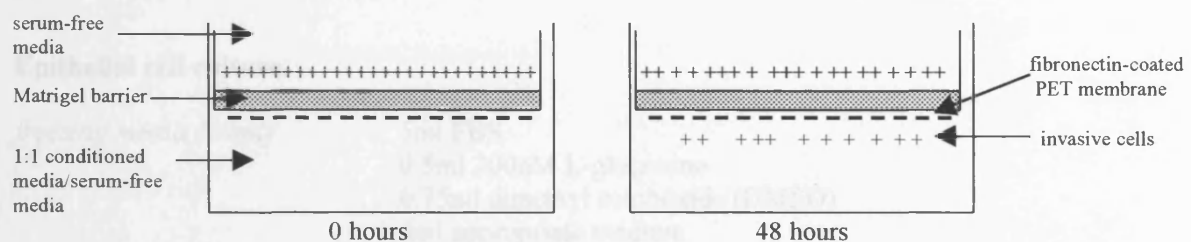
The inserts were removed and washed with PBS. Using a cotton bud, the cells were gently scraped off the top of one and the base of the other paired insert used in the invasion assay. The inserts were fixed in cold acetone for 10 minutes then placed in PBS overnight. Inserts were washed in tap water and stained with fresh haematoxylin for 2 minutes, rinsed in tap water, then stained with fresh eosin for 2 minutes and rinsed in tap water. Inserts were passed through

graded alcohols (95% alcohol 5 seconds, 99% alcohol 5 seconds, 99% alcohol 5 seconds), xylene for 30 seconds before the insert was cut out using a scalpel and mounted cells-down on a glass slide with DPX*.

The paired cell inserts were counted over 10 high power fields, each covering $0.25mm^2$, using an eye-piece graticule. The invasion index was calculated as follows:

$$\frac{\text{cells insert base} \times 100\%}{\text{cells insert top} + \text{cells insert base}}$$

Appendices*.

Immunohistochemistry.

Tris buffered saline (TBS 20x)	2.6mM NaCl, Tris 50mM
phosphate buffered saline (PBS 20x)	2.6M NaCl, 60mM Na_2HPO_4, 140mM NaH_2PO_4
citric acid buffer	10mM citric acid, pH 6.0
DAB solution	9ml de-ionised water 500μl diaminobenzidine tetrahydrochloride solution 500μl 20xTBS 100μl 3% H_2O_2
Mayers Haematoxylin	105mM aluminium potassium sulphate $(AlK(SO_4)_2.12H_2O)$ 5mM citric acid $(C_6H_3Cl_3O_2)$ 303mM chloral hydrate $(C_2H_3Cl_3O_2)$ 1mM sodium iodate $(NaIO_3)$
Eosin solution	1% aqueous water soluble eosin

Epithelial cell culture.

freezing media (10ml)	5ml FBS 0.5ml 200nM L-glutamine 0.75ml dimethyl sulphoxide (DMSO) 4ml appropriate medium

mRNA extraction.

oligo (dT)$_{25}$ Dynabeads

Supplied by Dynal as a suspension of 5mg beads in 1ml PBS, pH 7., and 0.02% NaN_3. In accordance with the manufacturers instructions 30μl beads (150μg) were used to extract mRNA from 10^5 cells.

Magnetic Particle Concentrator

MPC-E supplied by Dynal for isolation of Dynabeads. Holds up to 6 Eppendorf tubes with 8-10mm diameter and optimal working volume 0.5ml.

1M Tris-HCl pH 8.0

DEPC water was prepared by adding DEPC to ultra-pure (UP) water at a final concentration of 0.1% in a fume cupboard. This was shaken well and allowed to stand overnight. Autoclaving destroys any traces of DEPC. 12.1g tris base added to 80ml DEPC water in treated glassware. Dissolved and adjusted to pH 8.0. Made up to a final volume of 100ml with DEPC water. Autoclaved prior to use.

1.5M LiCl

0.636g LiCl added to 10ml PCR grade water and mixed well.

0.5M EDTA, pH 8.0

9.3g EDTA added to 40ml DEPC water. Adjusted to pH 8.0 and made up to 50ml final volume.

1% SDS

0.1g Lauryl sulphate added to 10ml PCR water and dissolved with gentle agitation.

Lysis-Binding Buffer (200ml)

	Stock Concentration	Vol. Stock	Final Concentration
Tris-HCl pH 8.0	1M	20ml	0.1M
LiCl	1.5M	67ml	0.5M
EDTA	0.5M	400µl	0.01M
SDS (w/v)	-	2g	1%
Dithiothreitol (DTT)	0.1M	10ml	0.005M

Made up to a final volume of 200ml with DEPC water and stored at 4°C.

Washing Buffer with SDS (200ml)

	Stock Concentration	Vol. Stock	Final Concentration
Tris-HCl pH 8.0	1M	2ml	0.01M
LiCl	1.5M	20ml	0.15M
EDTA	0.5M	40µl	0.001M
SDS (w/v)	-	0.2g	0.1%

Made up to a final volume of 200ml with DEPC water and stored at 4°C.

Washing Buffer (200ml)

	Stock Concentration	Vol. Stock	Final Concentration
Tris-HCl pH 8.0	1M	2ml	0.01M
LiCl	1.5M	20ml	0.15M
EDTA	0.5M	40µl	0.001M

Made up to a final volume of 200ml with DEPC water and stored at 4°C.

Reverse transcription.

5x AMV RT reaction buffer

This was supplied as 5x concentrate comprising:
   250mM Tris-HCl pH 8.3
   250mM KCl
   50mM $MgCl_2$
   50mM dithiothretiol (DTT)
   2.5mM Spermidine

AMV Reverse Transcriptase

Purified from avian myeloblastosis virus particles, supplied at 10U/µl.

dNTP mix

Supplied as stock solutions of 100mM. A mixture containing 10mM concentration each of dTTP, dCTP, dGTP and dATP was obtained by adding 10µl of stock solution of each dNTP to 60µl PCR grade water.

RnasIn

This is a recombinant ribonuclease inhibitor used as supplied.

Polymerase chain reaction.

10x AJ Buffer

	Stock Concentration	Vol. Stock	Final Concentration
Tris-HCl pH 8.0	1M	2250µl	45mM
$(NH_4)_2SO_4$	1M	550µl	11mM
$MgCl_2$	1M	225µl	4.5mM
each dNTP	100mM	100µl	200µM
bovine serum albumin	20mg/ml	275µl	110µg/ml
β Mercaptoethanol	14.3M	23.4µl	6.7mM
EDTA pH 8.0	0.01M	2.2µl	4.4µM

A final volume of 5ml was made by addition of 1274.4ml PCR grade water. This was dispensed into 100ml aliquots and stored at –20°C. Spinning for 3000-4000g was performed to remove precipitated protein.

Agarose gel electrophoresis.

Tris/Acetate/EDTA Buffer (TAE) (40mM Tris-acetate, 1mM EDTA)

Using analar grade reagents, a 50x stock solution was made up by dissolving 242g Tris base, 57.1ml glacial acetic acid and 100ml 0.5M EDTA pH8.0 in 600ml UP water. Adjusted to pH7.5-7.8. A final volume of 1000ml was made up with UP water and stored at room temperature.

For use the stock solution was diluted 1 in 50 with UP water.

Gel loading buffer

A 5x stock solution was made up and stored at room temperature.

20mg bromophenol blue
20mg xylene cyanol
2ml 50x TAE
10ml glycerol
8ml PCR-grade water

Invasion assays.

Fibroblast conditioned media

Human fetal foreskin fibroblasts (hfff2) were grown to 70-90% confluence in DMEM + 10% FBS. Media was removed and the cells were incubated in serum-free DMEM for 48 hours. This conditioned media was removed, aliquotted into eppendorfs and frozen at −80°C.

Growth factor-depleted Matrigel

This was batch-tested and the same batch was used for all experiments in this study.

Chapter 3

Microvessel Counts in
Operable Non-Small Cell Lung Cancer

Microvessel counts in Non-Small Cell Lung Cancer.

Introduction.

Angiogenesis is the formation of new blood vessels from the endothelium of the existing vasculature and is a requirement for solid tumour growth beyond 1-2mm in diameter (Folkman J, 1981). The regulation of angiogenesis is complex and is dependent on the balance between stimulatory and inhibitory factors. Evidence suggests that these factors include growth factors and their receptors, regulators of apoptosis and proteases and their inhibitors. Several retrospective and prospective studies in NSCLC have demonstrated that angiogenesis, assessed indirectly by microvessel counting, is an important prognostic factor, with high microvessel counts being associated with disease spread and a poor survival (Macchiarini P et al, 1992)(Giatromanolaki A et al, 1996a)(Fontanini G et al, 1997a). Evaluating the relationship between the microvessel count and the expression of these proposed regulatory factors in NSCLC tumour tissue may add insight into the metastatic process and identify potential targets that can be manipulated for therapeutic gain.

Thrombocytosis is associated with a worse prognosis in solid tumours including primary lung cancer (Paesmans M et al, 1995)(Pedersen LM et al, 1996). Angiogenic factors including vascular endothelial growth factor (VEGF) are transported by platelets (Verheul HMW et al, 1997) and released when platelets are activated (Mohle R et al, 1997). Platelet count has been shown to be associated with serum VEGF concentration in solid tumours (Verheul HMW et al, 1997)(O'Byrne KJ et al, 1999)(Salgado R et al, 1999). VEGF is the most potent and specific growth factor for endothelial cells and plays an important role in angiogenesis (Dvorak HF et al, 1995). VEGF also increases vascular permeability allowing leakage of plasma proteins, fibrin and platelets into the extracellular matrix (Dvorak HF et al, 1995). Recently it has been proposed that platelets contribute to tumour-induced angiogenesis by locally releasing angiogenic growth factors such as VEGF (Pinedo HM et al, 1998)(O'Byrne KJ et al, 1999).

The aim of this study was to evaluate microvessel counts in operable NSCLC and assess their impact on survival. This study also sought to evaluate the relationship between the microvessel count and the pre-operative platelet count and to allow the investigation of potential relationships with growth factor receptors, apoptotic regulators and matrix metalloproteinases in later chapters.

Methods.

This was a retrospective study of a consecutive series of patients with NSCLC who underwent surgical resection for stage I-IIIa disease in the Glenfield Hospital in Leicester and with post-operative survival > 60 days.

Immunohistochemistry:

This was performed as per methodology chapter using the monoclonal antibody to CD34 and streptavidin-biotin complex immunohistochemistry.

Vessel counting:

The anti-CD34 antibody was used as it gives more consistent and reproducible staining than von Willebrand factor and has far less cross reactivity with stromal and lymphatic endothelial cells (Hollingsworth HC et al, 1995). CD31 staining may also be variable due to cross reactivity with inflammatory and plasma cells (Goulding H et al, 1995). The anti-CD34 antibody has been shown to give more reliable and reproducible vascular staining than the anti-CD31 antibody (Martin L et al, 1997a). CD34 has previously been used to study microvessel counts in NSCLC (Fontanini G et al, 1997)(Matsuyama K et al, 1998).

Each section was examined under low power (x40, x100) to identify the three vascular 'hot spots' within the tumour (Weidner N et al, 1991). These areas were viewed under x250 by two investigators using a 25-point Chalkley eyepeice graticule (Fox SB et al, 1995). We considered as a vessel any structure that stained with the chromogen, irrespective of whether a lumen was present. Only vessels within one high-power field of viable tumour were deemed countable. The graticule was orientated so that the maximum number of points coincided with vessels. The total number of points from all three areas contributed to the Chalkley count. Microvessels adjacent to areas of unaffected lung parenchyma were omitted from the count (figure 3.1a). Due to deflation and compression after resection the vascular density in the unaffected lung may become falsely elevated. Sections were analysed in a blinded fashion and the results of the clinicopathological factors and outcome correlated subsequently. Figures 3.1b-d show examples of CD34 staining demonstrating an 'alveolar' pattern, a low microvessel count and a high microvessel count.

Studies evaluating semi-automated vessel counting by computerised image analysis have been used in an attempt to increase objectivity (Fox SB et al, 1995). The total section can be counted and the results are less dependent on the experience of finding the 'hot spot'. This study also attempted to use computerised colour-digital image analysis as a semi-automated method for

quantifying microvessel counts, vessel size and the total microvascular area in over 100 specimens. Initially the standard immuno- and counterstained sections were tried but colour-digital extraction of the brown-pigmented diaminobenzidine tetrahydrochloride (DAB)-immunostain from the blue Haematoxylin counterstain proved impossible. Finally red-pigmented 3-amino-9-ethylcarbazole (AEC) was used as an immunolabel and neutral green as a counterstain mounted with an aqueous mountant. Neutral green was slowly absorbed into the aqueous mountant until the detail of the section could not be seen. The rim of viable tumour was therefore marked on the coverslip with an indelible marker pen under light microscopy whilst the counterstain was still clearly visible. This method allowed digital image analysis of the red microvessels with no visible counterstain at a later date.

There were several further problems encountered with digital image analysis. Minor variations in immunointensity, easily identified by the eye, led to marked differences in the number and size of microvessels digitally recorded. There were inter and intra-section variations in lens to section distances for pin-point focussing. Minute variations in the accuracy of focussing also led to differences in scores. The light source intensity, camera and computer analysis system was set up for a single focal length so small changes in lens to section distances may alter the analysis. Using the computer was very labour-intensive as the whole of the tumour needed to be analysed to ensure identification of the hot spots. Sections frequently contained carbon in both lymphatics and throughout the tumour. Digital image analysis of sections with carbon deposits consistently gave higher microvessel counts as the carbon was also counted despite colour-digital extraction. Due to the insurmountable problem with carbon-pigmentation digital analysis was abandoned as a viable method of quantifying microvessels in lung tumours.

Pre-operative platelet count:

The platelet count was identified from the computer print-out of the pre-operative full blood count in the clinical records. Samples had been collected in EDTA-coated blood-specimen bottles and measured using a Coulter STKR automated counter. The laboratory met the National External Quality Assessment Scheme and Clinical Pathology Accreditation criteria.

Statistics:

Statistical analysis was performed using the SPSS software system (SPSS for Windows Version 9.0). The Chi-squared test was used to analyse the associations between categorical clinicopathological variables. Cancer-specific survival curves were plotted using the Kaplan-Meier method and the log-rank test was used to assess the statistical significance of differences between groups. The joint effects of covariables that were significant at the 0.25 level in univariate analysis were further examined via Cox regression using a forward selection

procedure. The 0.05 level of significance was used for entering or removing a covariable from this model.

Results.

Clinicopathological findings:

A total of 181 consecutive patients were recruited to the study. The follow-up ranged from 24-108 months (median 39.9 months). A total of 98 subjects (55.1%) died from a recurrence of their primary lung cancer. The stage of the tumour was found to be prognostic (p=0.0001). Tumour spread to nodes was associated with poor prognosis (p=0.0007) and increasing nodal status was more significant (p<0.0001). No other clinicopathological finding, including age, sex, grade or histological sub-type was associated with outcome. The clinicopathological findings are listed in table 3.1.

Microvessel counts:

Staining for CD34 was specific with minimal background staining. Normal lung tissue demonstrated high vascularity. An 'alveolar' pattern was seen near the invading edge of several NSCLC tumours but tended to be lost towards the centre of the tumour. Vessel quantification was possible in 180 of the 181 cases. In one case marked stromal reactivity was seen precluding vessel counting. Intra-observer variability was analysed in all 180 cases (R=0.92; p<0.0001). Inter-observer variability was analysed in 62 cases and was minimal (R=0.78; p<0.0001). The mean Chalkley count was 18.2, SD 5.29, range 8-33 and the median Chalkley count was 18. A Chalkley count > median was associated with a poor survival (p=0.01) (figure 3.2a) as was the upper tertile ($\geq$21) (p=0.0006) (figure 3.2b). Outcome was worse for a Chalkley count in the upper tertile in all stages reaching statistical significance in stage I disease (p=0.02). A Chalkley count > median was associated with nodal disease (p=0.004) and with increasing nodal spread (p=0.007). There was no association between Chalkley count and age, grade or histological type.

Pre-operative platelet count:

The pre-operative platelet count was identified in 175 cases (mean 344×10^9/l, SD 122×10^9/l, median 320×10^9/l, range 92 to 910 $\times 10^9$/l). A platelet count greater than the median was associated with a poor outcome (p=0.01) (figure 3.2c). A platelet count above the normal range (>400 $\times 10^9$/l) was present in 48 (26.5%) subjects and correlated with a worse prognosis (p=0.03) (figure 3.2d). There was no association between platelet count and the subject age, sex, nodal status, grade or histological sub-type (table 3.2).

Table 3.1 Prognostic significance of tumour variables (n=181).

prognostic factor	no.	%	log-rank
Age- (years)			
mean-	64.5 (SD 7.57)		
median-	66 years		p=0.62
range-	42-78 years		
Sex-			
male-	128	70.7%	
female-	53	29.3%	p=0.19
Histology-			
squamous-	113	62.4%	
adenocarcinoma-	54	29.8%	p=0.96
large cell-	14	7.7%	
Grade-			
well/moderate-	92	50.8%	
poorly differentiated-	89	49.2%	p=0.36
Stage-			
I-	88	48.6%	
II-	51	28.2%	p=0.0001
IIIa-	42	23.2%	
T-			
1-	33	18.2%	
2-	129	71.3%	p=0.22
3-	19	10.5%	
N-			
0-	93	51.4%	
1-	56	30.9%	p<0.0001
2-	32	17.7%	
Platelet count- (n=175)(x10^9/l)	mean 344 (SD 122)		median 320 (range 92-910)
>median-	86	49.1%	
≤median-	89	50.9%	p=0.01
Chalkley count- (n=180)	mean 18.2 (SD 5.29)		median 18 (range 8-33)
>median-	86	47.5%	
≤median-	95	52.5%	p=0.01
upper tertile (≥21)-	67	37.0%	
lower tertiles (<21)-	114	63.0%	p=0.0006

Table 3.2 Relationships with platelet count (n=175).

prognostic factor	platelet count		χ^2
	below median	above median	
Age-			
below median-	38	47	
above median-	50	40	0.15
Sex-			
male-	63	60	
female-	25	27	0.70
Histology-			
squamous-	51	57	
adenocarcinoma-	28	25	0.44
large cell-	9	5	
Grade-			
well/moderate-	40	49	
poorly differentiated-	48	38	0.15
Stage-			
I-	48	37	
II-	21	28	0.27
IIIa-	19	22	
T-			
1-	20	13	
2-	61	63	0.30
3-	7	11	
N-			
0-	50	40	
1-	22	31	0.27
2-	16	16	
Chalkley count- (n=174)			
below median-	45	46	
above median-	43	40	0.76
lower + middle tertile-	53	57	
upper tertile-	35	29	0.41

72

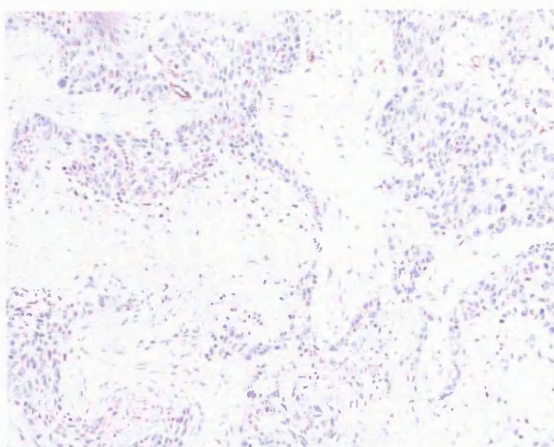# Figure 3.1 Immunohistochemistry for CD34 staining.

a) 'normal' lung adjacent to tumour
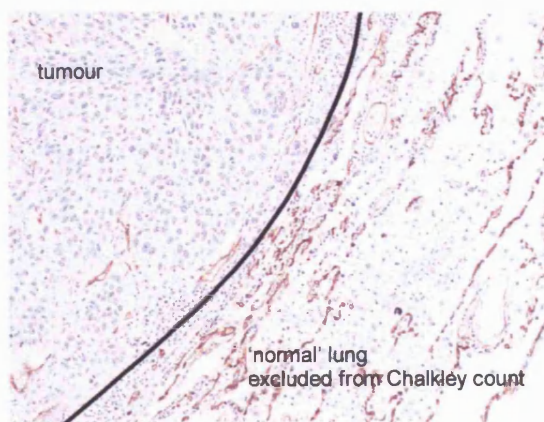
b) alveolar pattern
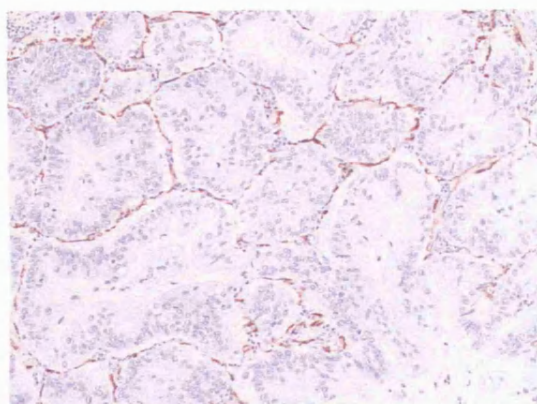
c) CD34 low micovessel count
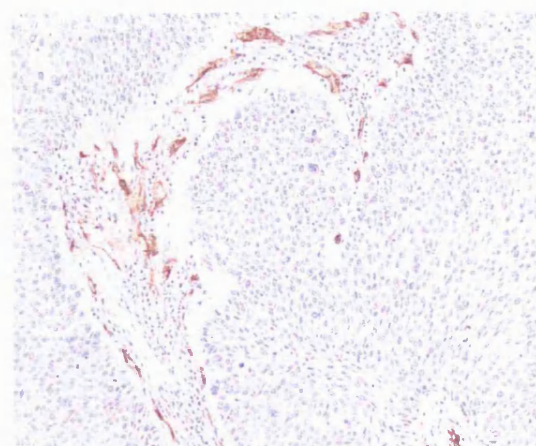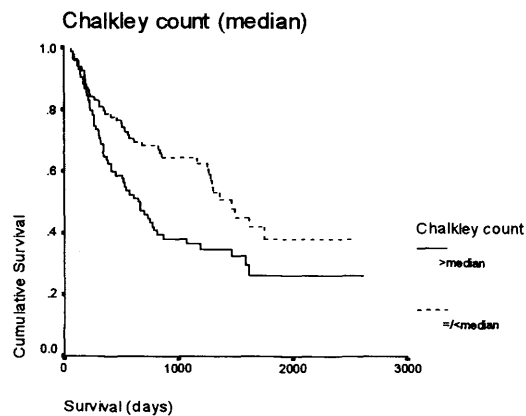

d) CD34 high microvessel count

Figure 3.2 Survival curves for microvessel counts and pre-operative platelet counts in operable NSCLC and a scatter plot of their interrelationship.

figure 3.2a:

Chalkley count (median)

p=0.01

figure 3.2b:

Chalkley count (high vs medium/low)

p=0.0006

figure 3.2c:

Pre-Operative Platelet Count

p=0.01

figure 3.2d:

Pre-operative Platelet Count
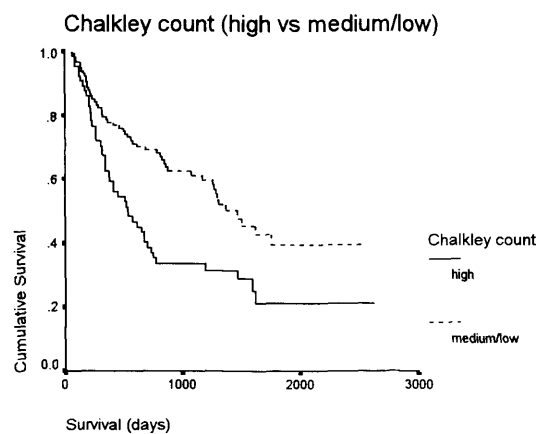
p=0.03

figure 3.2e:
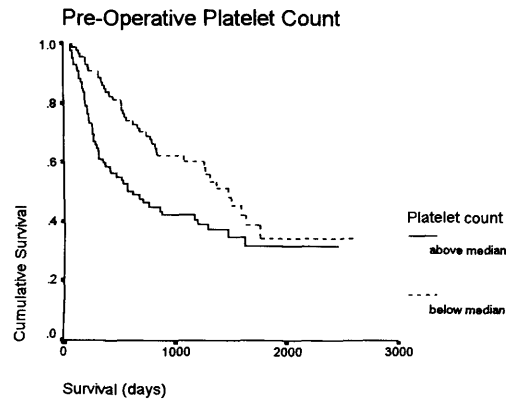
Chalkley count vs pre-operative platelet count
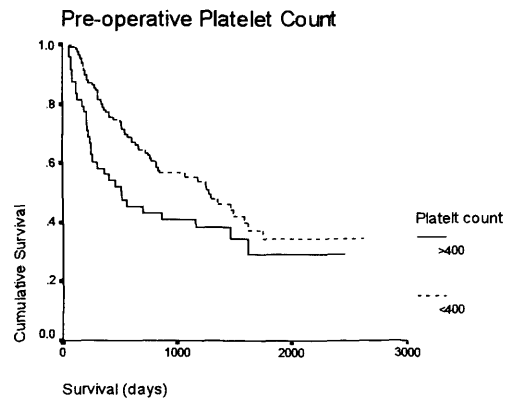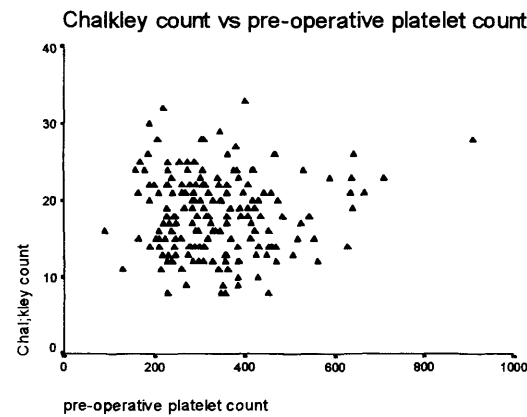
R=0.05 p=0.56

Microvessel counts and platelet counts:

There was no correlation between the pre-operative platelet count and the Chalkley count (figure 3.2e) (R=0.05 p=0.56).

Multivariate analysis:

Cox proportional hazards regression analysis was used to define biological markers with independent predictive value with respect to cancer-specific survival (table 3.3). The most significant independent prognostic factor was nodal status. The addition of Chalkley count and pre-operative platelet count produced a statistically significant improvement in the model.

Table 3.3 Multivariate analysis.

factor		hazard ratio	95% confidence interval	p-value
nodal status-				
	0-	1.00		0.0009
	1-	1.56	0.97-2.51	
	2-	2.70	1.60-4.56	
Chalkley count-				
low/medium-		1.00		0.005
high-		1.80	1.19-2.73	
platelet count-				
below median-		1.00		0.01
above median-		1.69	1.12-2.54	

Discussion.

The growth of a tumour beyond 1-2mm in diameter requires the development of new blood vessels (Folkman J, 1981). These vessels play an important role in tumorigenesis supplying nutrients and oxygen, disposing of metabolic waste products and allowing metastatic spread. The present study showed that tumours with a high vessel count were associated with a worse survival. This is in agreement with several other studies showing an inverse relationship between vascularity and patient survival in NSCLC (Macchiarini P et al, 1992)(Yamasaki K et al, 1994)(Fontanini G et al, 1995a)(Angelletti CA et al, 1996)(Giatromanolaki A et al, 1996a)(Harpole DH et al, 1996)(Fontanini G et al, 1997a)(Kanaguchi T et al, 1997)(Duarte IG et al, 1998)(Matsuyama K et al, 1998). Anti-CD34 antibody was used as a specific endothelial cell marker and this antibody has been used in NSCLC previously (Fontanini G et al, 1997a)(Chandrachud LM et al, 1997)(Dazzi C et al, 1999)(Decaussin M et al, 1999)(Yano T et al, 2000). Chalkley counting was the chosen microvessel quantification method and this has been validated in both breast (Imai T et al, 1991)(Fox SB et al, 1995) and NSCLC studies (Giatromanolaki A et al, 1996a)(Decaussin M et al, 1999). Multivariate analysis revealed

Chalkley count to be the most important prognostic indicator behind nodal status. This adds more evidence towards the importance of angiogenesis in tumour growth and spread.

However, some investigators have not demonstrated a prognostic influence from vessel counts (Pastorino U et al, 1997)(Chandrachud LM et al, 1997)(Apolinario RM et al 1997). A number of factors may account for the findings in these studies. Different antibodies and counting techniques are used in vessel quantification studies. The sensitivity of microvessel counting is dependent upon the experience of the investigators (Vermeulen PB et al, 1997). Several studies have shown a correlation between microvessel count and stage therefore studying single stage disease may lead to a negative result (Fontanini G et al, 1995)(Giatromanolaki A et al, 1996a). Our study population included all stages of resectable NSCLC (stage I-IIIa). In each individual stage a Chalkley count in the upper tertile conferred a worse outcome reaching significance in stage I disease. A recent study in NSCLC has demonstrated heterogeneity of vasculature within blocks from the same region of the tumour and shown that the periphery of the tumour does not always contain the highest number of vessels. Despite these variations, a two-tier vascularity ranking system produced correct ranking in around 70% of randomly selected sections, and was even higher in sections taken from the periphery (Schor AM et al, 1998). However, vessel counting from a single breast tumour section has been shown to correlate with whole tumour vascularity as measured by microangiography (Martin L et al, 1997b).

An 'alveolar' pattern of endothelial staining has been thought to show a tumour exploiting the existing vasculature for its requirements without the need for angiogenesis (Pezzella F et al, 19997). This pattern was seen at the invasive edge of several tumours but was lost towards the centre of the tumour. This could be due to the tumour initially being able to survive on the supply of nutrients from the existing vascular bed before angiogenic factors released from the tumour and tumour-induced stromal remodelling disrupt this pattern.

This study was unsuccessful in validating a semi-quantitative computer image analysis as a reliable method of microvessel quantification. The insurmountable problem was the presence of carbon deposits in many tumour sections that were then incorrectly identified as microvessel-staining. Other solid tumours, for instance breast carcinoma (Fox SB et al, 1995), have been assessed using digital analysis but they do not have the same problem with carbon pigmentation.

A pre-operative platelet count above the median was associated with a poor prognosis in this study. A platelet count above the normal range (>400 x10^9/l) was also associated with a worse outcome. The cause of thrombocytosis in cancer patients is not well understood although interleukin-6 (IL-6) has been implicated. IL-6 is a potent stimulator of platelet proliferation

(Imai T et al, 1991), is released by primary tumours and their metastases (Watson HM et al, 1990)(Salgado R et al, 2000) and is upregulated by hypoxia (Yan SF et al, 1995).

Recently the hypothesis that platelets may be mediators of VEGF-induced angiogenesis has been proposed (Pinedo HM et al, 1998)(O'Byrne KJ et al, 1999). Platelet count has been shown to correlate with both serum VEGF (Pinedo HM et al, 1998)(Salgado R et al, 1999) and IL-6 (Salgado R et al, 1999) levels in patients with solid tumours. IL-6 levels were also found to correlate with the VEGF load per platelet (VEGF^{PLT}) (Salgado R et al, 1999) and increased VEGF^{PLT} levels have been associated with poor prognosis in renal cell carcinoma (O'Byrne KJ et al, 2000a). Platelet adherence to tumour vessels may be increased by low blood-flow in tumours and/or the increased presentation of platelet-binding proteins either through stromal remodelling, microvessel hyperpermeability or the release of von Willebrand factor (Brock TA et al, 1991). These conditions make it more likely that platelets will adhere to tumour microvessels where they can release pro-angiogenic growth factors such as VEGF (Dvorak HF et al, 1995).

There was no correlation between the pre-operative platelet count and the Chalkley count in this study. There are several reasons why this may be the case. The circulating platelet count may not indicate the local platelet numbers adherent within a tumour where the local release of angiogenic factors from platelets may still be important. Platelets may also release inhibitors of angiogenesis such as thrombospondin (Iruela-Arispe ML et al, 1991) and platelet factor 4 (Maione TE et al, 1990). The balance between pro- and anti-angiogenic factors released locally within the tumour by platelets may contribute to the regulation of neovascularization. The production of angiogenic growth factors produced by the tumour itself may be more important. This is supported by studies showing a correlation between microvessel count and both tumour cell VEGF (Fontanini G et al, 1997b)(Tsao MS et al, 1997)(Giatromanolaki A et al, 1998a) and platelet-derived endothelial cell growth factor (PD-ECGF) expression (Koukourakis MI et al, 1997a). Nonetheless this does not exclude a role for platelets in the angiogenic process in solid tumours.

Chapter 4

Regulators of Apoptosis and Growth Factor Receptors in Operable Non-Small Cell Lung Cancer

Regulators of Apoptosis and Growth Factor Receptors and in Operable Non-Small Cell Lung Cancer.

Introduction.

Neoplastic transformation is brought about through the activation of oncogenes or the inactivation of tumour suppressor genes. Tumour growth is dependent on the balance between increasing cell numbers through proliferation and reduction in cell numbers through apoptosis and necrosis.

An alteration in either the p53 gene or protein is the most common change in human malignancy. p53 causes arrest of the cell cycle at the G_1-phase, increases DNA repair time, decreases replicative DNA synthesis and induces apoptosis. Mutations of the p53 gene enable a cell to survive longer under hypoxic conditions (Graeber TG et al, 1996). Loss of p53 expression leads to increased angiogenesis by decreasing the 170kDa matrix glycoprotein thrombospondin-1 (Dameron KM et al, 1994). As p53 has a short half-life, immunostaining for p53 identifies protein over-expression or more usually a stable mutant protein. p53 expression is found in pre-invasive lesions of the bronchial tree suggesting mutation is an early event in the process of malignant transformation leading to lung cancer (Sozzi G et al, 1992)(Bennett WP et al, 1993). The evidence of a prognostic role for p53 expression in NSCLC is contradictory. p53 expression has been shown to be related to a good prognosis (Lee JS et al, 1995)(Passlick B et al, 1995)(Top B et al, 1995), and a poor prognosis (Quinlan DC et al, 1992)(Horio Y et al, 1993)(Harpole DH et al, 1995a)(Dalquen P et al, 1995)(Fukuyama Y et al, 1997)(Fontanini G et al, 1997c) or to have no prognostic significance (McLaren R et al,1992)(Nishio M et al, 1996).

Bcl-2 is a member of a family of genes involved in the regulation of apoptosis. The Bcl-2 gene differs from conventional oncogenes as it can neither promote growth nor directly lead to cellular transformation. The Bcl-2 gene codes for an integral membrane protein localised to the cytoplasm on the outer nuclear and mitochondrial membranes and the endoplasmic reticulum where it is thought that Bcl-2 acts as a transmembrane protein and ion channel, and as a docking protein (Minn AJ et al 1998). Bcl-2 is capable of binding p53 and preventing its passage from the cytoplasm to the nucleus (Ryan JJ et al, 1994)(Naumovski L et al, 1996). Increased levels of Bcl-2 expression prevent apoptosis from a wide range of insults including growth factor depletion, ionizing irradiation and chemotherapeutic regimes (Nunez G et al, 1990)(Miyashita T et al, 1992) (Lotem J et al, 1993). As Bcl-2 inhibits apoptosis, Bcl-2 over-expression should lead to proliferation, favour the malignant process and result in a poor outcome. Paradoxically

Bcl-2 expression is associated with improved survival in various solid tumours including breast and endometrial cancer (Silvestrini R et al, 1996)(Sakuragi N et al, 1998).

The erb/HER type I tyrosine kinase receptor family mediates proliferation and differentiation in normal epithelial cells. Inappropriate over-expression may contribute to tumour growth and invasion (Walker RA, 1998). Epidermal growth factor receptor (EGFR) and c-erbB-2 (HER-2 or neu) are two important members of this family. EGFR expression is generally low in normal bronchial epithelium and is enhanced in preneoplastic and neoplastic lesions (Hendler FJ et al, 1984). EGFR expression has been shown to be associated with lymph node spread (Fontanini G et al, 1995c) and poor prognosis (Veale D et al, 1993)(Harpole DH et al, 1996)(Pastorino U et al, 1997)(Ohsaki Y et al, 2000) in NSCLC in some studies but not in others (Pfieffer P et al, 1996)(Rusch V et al, 1997).

Over–expression of c-erbB-2 has been demonstrated in many epithelial tumours including breast cancer (Wright C et al, 1989)(Press MF et al, 1997). Previous studies of c-erbB-2 expression in NSCLC vary in antibodies used and immunohistochemical technique employed as well as in the definition of positive cases. Studies on paraffin sections vary in reports on membranous and cytoplasmic expression. Membranous staining has been demonstrated in 2-40% of NSCLC cases (Kern JA et al, 1990)(Pastorino U et al, 1997) and found to be associated with a poor prognosis for adenocarcinoma (Kern JA et al, 1990). Other studies have reported cytoplasmic immunostaining in 10-60% of NSCLC cases (Weiner DB et al, 1990)(Tateishi M et al, 1991)(Harpole DH et al, 1995a)(Giatromanolaki A et al, 1996b)(Fontanini G et al, 1998a)(Koukourakis MI et al, 1999). Although only membranous expression would be expected to be functionally important, several of these studies have shown cytoplasmic c-erbB-2 expression to be associated with a poor prognosis (Weiner DB et al, 1990)(Tateishi M et al, 1991)(Harpole DH et al, 1995a) especially in poorly vascularised tumours (Koukourakis MI et al, 1999). One study has shown that NSCLC tumours containing higher levels of c-erbB-2 protein have a worse outcome (Diez M et al, 1997). Another study showed c-erbB-2 expression in 17/138 (12%) of localised lung adenocarcinomas with only one of these immunopositive cases demonstrating gene amplification (Harpole DH et al, 1995b). This suggests that, unlike breast carcinoma, c-erbB-2 gene amplification in NSCLC is uncommon.

Over-expression of the c-erbB-2 on the surface of a tumour cell makes this protein a potential target for the recently developed anti-c-erbB-2 monoclonal antibody construct Herceptin™ (Cobleigh MA et al, 1999). Herceptin™ enhances the anti-tumour activity of cytotoxic chemotherapeutic agents and significantly prolongs survival in metastatic breast carcinoma

(Baselga J et al, 1998)(Slamon D et al, 1998). Potentially NSCLC tumours over-expressing c-erbB-2 may also be treated with Herceptin™.

The HercepTest™ was designed as a semi-quantitative immunohistochemical assay to determine c-erbB-2 protein over-expression in routinely processed breast cancer tissue to aid the assessment of patients for whom c-erbB-2 antibody treatment with Herceptin™ is being considered (Graziano C, 1998)(Jacobs TW et al, 1999).

This study sought to evaluate any interrelationships between p53, Bcl2, EGFR and c-erbB-2 expression in NSCLC and to assess the impact of these factors on survival.

Methods.

This was a retrospective study of patients with NSCLC who underwent surgical resection for stage I-IIIa disease in the Glenfield Hospital in Leicester, UK and with post-operative survival > 60 days. For the HercepTest™, resected cases with survival < 60 days and bronchoscopic, open and percutaneous lung biopsies for stage IV disease NSCLC were also evaluated. A further 94 consecutive NSCLC resection cases (excluding large cell carcinoma) were evaluated for c-erbB-2 expression by Moygens Vyberg and Brenda Melgaard in the Institute of Pathology, Aalborg University Hospital, Aalborg, Denmark.

Immunohistochemistry:
This was performed as per methodology chapter using antibodies to p53, Bcl-2 and EGFR and standard ABC immunohistochemistry. Immunohistochemistry for c-erbB-2 was performed using the HercepTest™. Furthermore, 70 sections from Leicester resection cases, including all positive cases, were sent to Dako, Glostrup, Denmark for independent evaluation.

During the optimisation of the immunohistochemical technique a positive control tissue was included for which the pattern of reactivity with each antibody was known. Evaluation of this section with each immunohistochemistry run ensured that the appropriate staining pattern was achieved. All negative controls were negative.

Evaluation of immunohistochemistry:
The extent and pattern of reactivity for p53, Bcl-2, EGFR and c-erbB-2 was recorded by two observers. The extent of expression for nuclear and cytoplasmic p53 staining, cytoplasmic Bcl-2 staining and membranous and cytoplasmic EGFR staining was scored 0 for no staining, <20%, 20-50% and 50-100%. A similar semi-quantitative scale of 0, +, ++ or +++ was used to assess

the intensity of staining in comparison to a known positive control. Cases were called positive if staining intensity was ++ or +++ over at least 20% of the tumour. The HercepTest™ scoring system was applied for c-erbB-2 evaluation. Cases scored 0 for no staining or membranous staining in <10% of the tumour; 1+ for faint staining of >10% of the tumour cells and staining of only part of the membrane; 2+ for weak or moderate complete membrane staining in >10% of the tumour cells and 3+ for strong complete membranous staining in >10% of tumour cells. Cases scoring 2+ or 3+ were deemed positive and cases scoring 0 or 1+ were deemed negative. Sections were analysed by two observers in a blinded fashion and the results of the immunohistochemistry, tumour status and patient outcome correlated subsequently.

Fluorescence in-situ hybridisation:

Fluorescence in-situ hybridisation (FISH) analysis for the c-erbB-2 gene was performed on a total of 28 NSCLC tumours with a selection of 0, 1+, 2+ and 3+ HercepTest™ scores by Moygens Vyberg and Brenda Melgaard in the Institute of Pathology, Aalborg University Hospital, Aalborg, Denmark as per methods chapter.

Amplification was defined as a ratio of c-erbB-2 signals (red dots) to chromosome 17 centromere signals (green dots) >2. Polyploidy was defined as a mean of >4 chromosome 17 centromere signals per cell.

Statistical analysis:

Statistical analysis was performed using SPSS for Windows version 9.0. Survival curves were plotted using the methods of Kaplan-Meier, and the log-rank test was used to assess the significance of statistical differences between groups. Chi squared-tests were used to assess associations between categorical variables. Inter-observer variation was assessed using Kappa statistics. The influence of clinicopathological factors including p53, Bcl-2, EGFR and c-erbB-2 immunoexpression on overall survival were assessed by the Cox proportional hazards regression model. Statistical significance was determined using a 5% significance level.

Results.

A total of 181 patients who had undergone surgical resection for stage I-IIIa NSCLC in Leicester with a post-operative survival greater than 60 days were used for prognostic data (previous chapter table 3.1). Sections from a further 117 patients who underwent resection (23 in Leicester, 94 in Aalborg) and 46 who underwent bronchoscopic or percutaneous biopsy were also stained for c-erbB-2. The results of the immunohistochemistry are set out in table 4.1.

Table 4.1 Prognostic significance of immunohistochemistry for p53, Bcl-2, EGFR and HercepTest™ (n=181).

Prognostic factor		No.	%	log-rank survival
p53-	negative-	95	52.5%	
	positive-	86	47.5%	p=0.43
	nuclear-	78	43.1%	p=0.29
	cytoplasmic-	20	11.0%	p=0.26
Bcl-2-	negative-	116	65.2%	
(n=178)	positive-	62	34.8%	p=0.04
	weak-	43	24.2%	
	strong-	19	10.7%	
			negative v weak	p=0.006
			negative v strong	p=0.57
			weak v strong	p=0.01
EGFR-	negative-	84	46.4%	
	positive-	97	53.6%	p=0.58
	membranous-	57	31.5%	p=0.35
	cytoplasmic-	40	22.1%	p=0.72
HercepTest™-	negative-	178	98.7%	
	positive- (2+ or 3+)	3	1.7%	p=0.62

p53 immunostaining:

p53 immunostaining was performed in all cases. p53 immunoreactivity was usually found in the nucleus of tumour cells (figure 4.1a) and occasionally in the cytoplasm. 43.1% (78/181) demonstrated nuclear positivity and 11.0% (20/181) cytoplasmic reactivity. Both nuclear and cytoplasmic staining was demonstrated in 11 cases (6.2%). No correlation was found between p53 and T status or grade. Cases with N_2 disease were more likely to be p53 negative (p=0.01). Both p53 and nuclear p53 immunoreactivity were more frequent in squamous cell carcinoma than in other histological subtypes (p=0.003 and <0.001 respectively). Neither nuclear p53 (figure 4.2a) nor cytoplasmic p53 (figure 4.2b) reactivity correlated with survival (p=0.29 and p=0.26 respectively).

Bcl-2 immunostaining:

Bcl-2 immunostaining was performed in 178 cases. Bcl-2 immunostaining was often detected in the basal layer of normal bronchial epithelium (figure 4.1b). Bcl-2 immunoreactivity was localised to the cytoplasm and the perinuclear region (figures 4.1c and 4.1d). Staining of lymphocytes was used as an internal control. Any case with >20% tumour cells showing immunostaining were evaluated as positive. Bcl-2 positivity was detected in 62 out of 178

(34.9%) cases. Bcl-2 immunopositivity was more common in squamous cell carcinoma cases than in other histological types (p=0.01). There was no correlation between Bcl-2 immunoreactivity and any other clinicopathological parameter. A sub-group of 19 strongly immunostaining cases (10.7%) was identified where intracellular and perinuclear membrane staining was intense (inter-observer correlation R=0.94 p<0.0001). Overall Bcl-2 immunopositivity was an indicator of good prognosis (p=0.04)(figure 4.2c). The subset of strongly staining cases had a significantly worse prognosis than cases where the staining was weak (p=0.01) and the prognosis of weakly staining cases was significantly better than negative cases (p=0.006)(figure 4.2d).

Of 62 Bcl-2 positive tumours, 30 (48.4%) showed nuclear p53 expression compared to 47 out of 116 Bcl-2 negative tumours (40.5%) (non-significant).

EGFR immunostaining:

EGFR immunostaining was performed in all cases. Immunoreactivity for EGFR was present in 97/181 (54%) of tumours. Membranous immunopositivity with or without cytoplasmic staining was seen in 57/181 (32%) of cases (figure 4.1e). Cytoplasmic expression without membranous staining was seen in 40/181 (22%) of cases (figure 4.1f). Large cell and squamous cell carcinomas more frequently expressed EGFR than adenocarcinomas (p<0.0001) and expression was more common in the elderly (p=0.007). Membranous EGFR expression was more frequent in squamous cell carcinomas (p=0.01), in the elderly (p=0.02), in poorly differentiated tumours (p=0.03) and in males (p=0.05). Membranous (figure 4.2e), cytoplasmic (figure 4.2f) and overall EGFR expression were not associated with outcome (p=0.35, p=0.72 and p=0.58 respectively).

There was no association demonstrated between p53 and overall or membranous EGFR expression. Cases with immunoexpression for both p53 and overall EGFR and membranous EGFR expression showed a non-significant trend towards a worse outcome (p=0.11 and p=0.19 respectively).

HercepTest™ immunostaining:

HercepTest™ immunostaining was performed in all 181 resection cases from Leicester as well as 23 cases with either a post-operative survival <60 days or pathologically proven stage IV disease. A further 94 resection cases from Aalborg and 46 biopsies from patients with stage IV disease were also stained. The clinicopathological findings and immunohistochemistry for all these cases are set out in table 4.2. HercepTest™ immunohistochemistry highlighted membranous, but not cytoplasmic, tumour cell staining (figure 4.1g). Occasional cases

Table 4.2 Clinicopatholocal findings for HercepTest™.

		Leicester resections		Aalborg resections		Leicester biopsies		overall	
Number		204		94		46		344	
Sex	male-	146	(72%)	63	(67%)	34	(74%)	243	(71%)
	female-	58	(28%)	31	(33%)	12	(26%)	101	(29%)
Histology	squamous-	129	(63%)	47	(50%)	12	(26%)	188	(55%)
	adenocarcinoma-	59	(29%)	47	(50%)	15	(33%)	121	(35%)
	large cell-	16	(7.8%)	0	(0%)	0	(0%)	16	(4.7%)
	'NSCLC'-	0	(0%)	0	(0%)	19	(41%)	19	(5.5%)
Grade	well/moderate-	100	(49%)	49	(52%)	7	(17%)	156	(45%)
	poorly differentiated-	104	(51%)	45	(48%)	39	(83%)	188	(55%)
Stage	I-	94	(45%)	50	(53%)	0	(0%)	144	(42%)
	II-	57	(28%)	20	(21%)	0	(0%)	77	(22%)
	III-	49	(24%)	22	(23%)	0	(0%)	71	(21%)
	IV-	4	(2.0%)	2	(2.1%)	46	(100%)	52	(15%)
Herceptest™	0-	181	(89%)	77	(82%)	33	(72%)	291	(85%)
	1+-	17	(8.3%)	13	(14%)	8	(17%)	38	(11%)
	2+-	4	(2.0%)	2	(2.1%)	2	(4.3%)	8	(2.3%)
	3+-	2	(1.0%)	2	(2.1%)	3	(6.5%)	7	(2.0%)

Pathological stage of positive cases
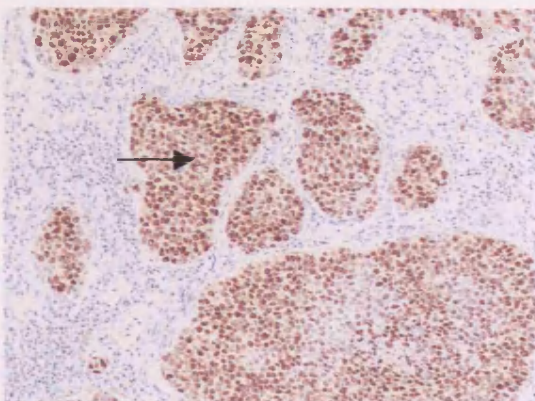
2+ 8 cases: Ia, Ia, Ib, Ib, IIb, IIb, IV, IV

3+ 7 cases: IIb, IIIa, IV, IV, IV, IV

association of HercepTest™ positivity with higher stage p=0.001
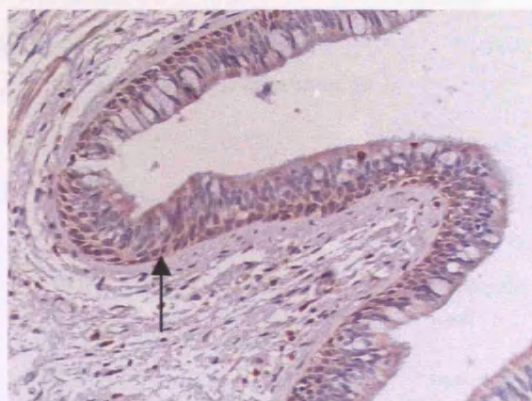
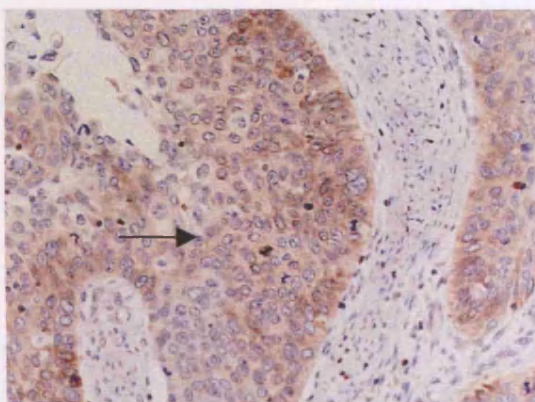# Figure 4.1 Immunohistochemistry for p53, Bcl-2, EGFR and c-erbB-2 staining.

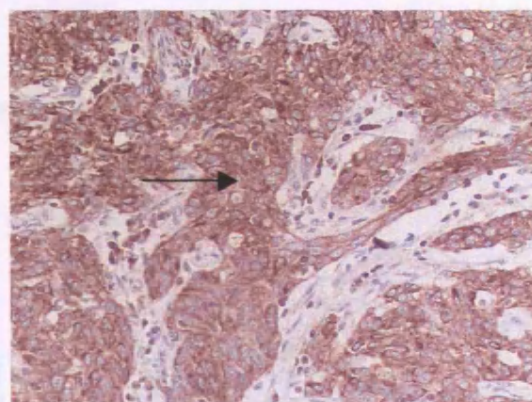a) nuclear p53 staining in tumour cells

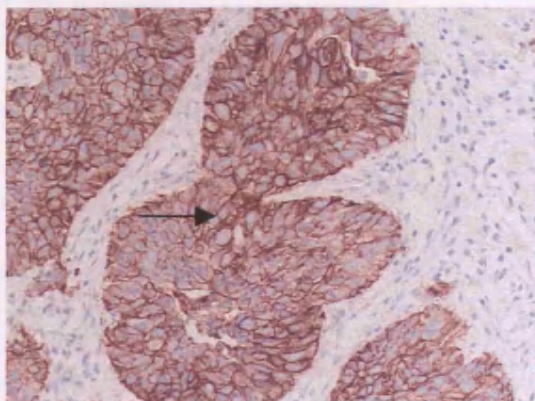b) Bcl-2 staining in basal respiratory epithelial cells

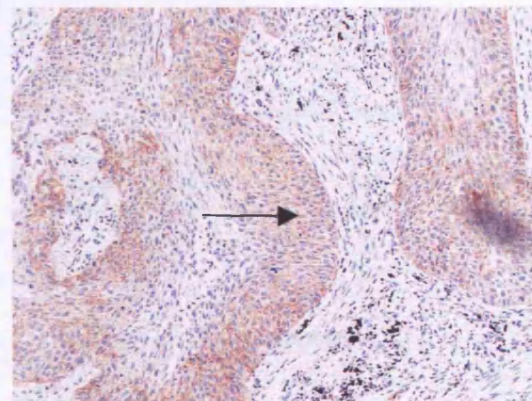
c) weak Bcl-2 tumour cell staining
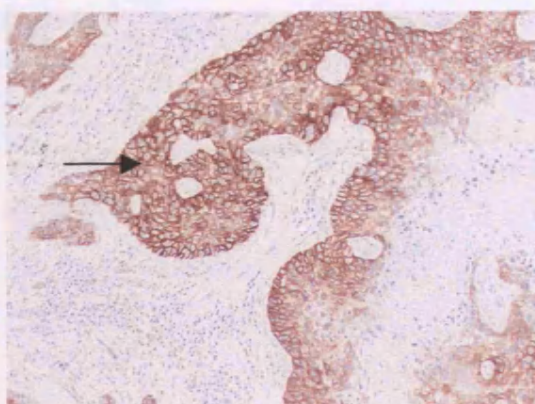
d) strong Bcl-2 tumour cell staining

e) membranous EGFR tumour cell staining

f) cytoplasmic EGFR tumour cell staining

h) HercepTest™ 3+ strong membranous staining

i) single positive HercepTest™ 'clones'
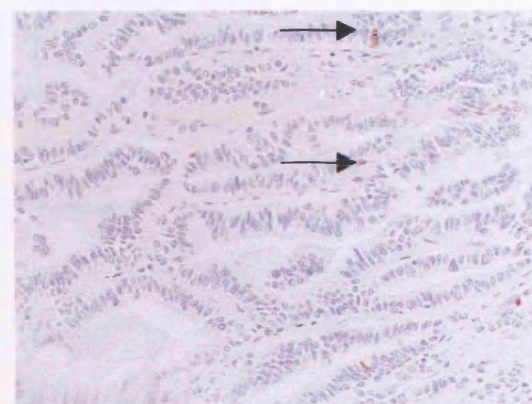
Figure 4.2 Survival curves for p53, Bcl-2, EGFR and c-erbB-2

figure 4.2a:

nuclear p53 expression

p=0.29

figure 4.2b:

cytoplasmic p53 expression

p=0.26

figure 4.2c:

Bcl-2 expression

p=0.04

figure 4.2d:

Bcl-2 immunointensity
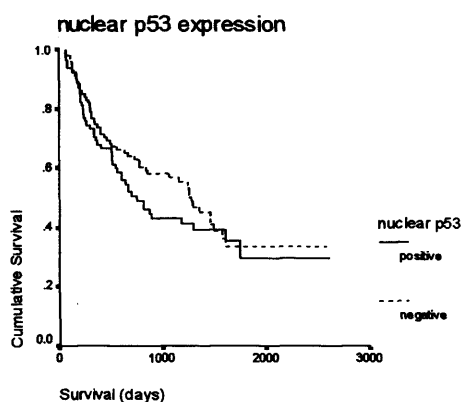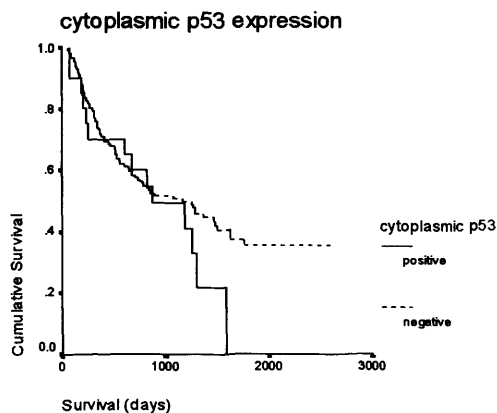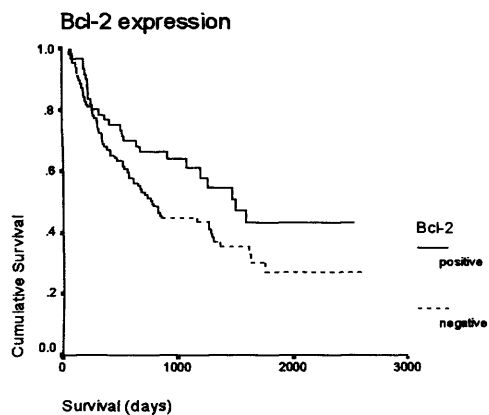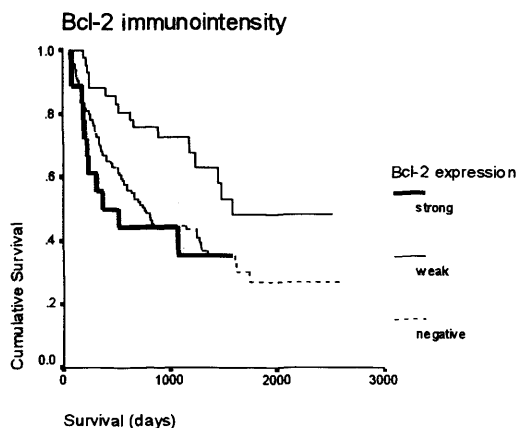
overall	p=0.01
negative vs weak	p=0.006
negative vs strong	p=0.57
weak vs strong	p=0.01

figure 4.2e:

membranous EGFR expression

p=0.35

figure 4.2f:

cytoplasmic EGFR expression
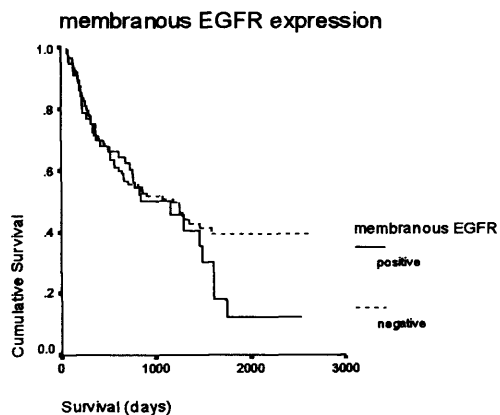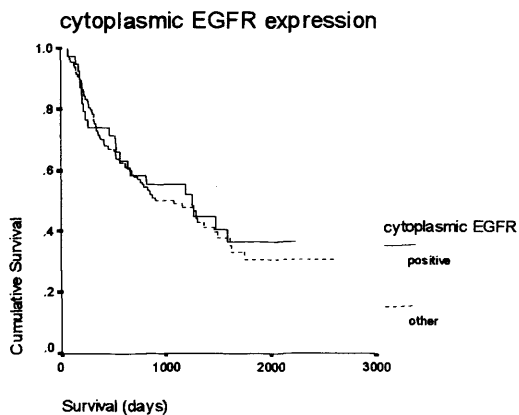
p=0.72

demonstrated single tumour cells with strong cytoplasmic staining (figure 4.1h). Overall HercepTest™ positivity was found in 15/344 (4.3%) NSCLC cases assessed (8 cases 2+, 7 cases 3+). There was no correlation between HercepTest™ positivity and tumour grade or histology. In stage I disease 4/144 (2.8%) cases were positive, 3/77 (3.9%) in stage II, 1/71 (1.4%) in stage III and 7/52 (13.5%) in stage IV. HercepTest™ positive cases were associated with advanced stage (p=0.001) with 5/7 3+ cases being stage IV disease. All positive cases in Leicester gave identical immunostaining scores when stained by Dako in Denmark.

c-erbB-2 FISH analysis:

FISH analysis revealed tumours to either demonstrate no amplification, no amplification but polyploidy, or amplification for the c-erbB-2 gene. The results of the FISH analysis and comparison with the HercepTest™ score are outlined in table 4.3. All the 3+ cases tested demonstrated amplification (figure 4.3a). Areas of borderline gene amplification and areas of polyploidy were demonstrated in 1 out of the 5 2+ cases tested (figure 4.3b). No HercepTest™ negative cases (0 or 1+) demonstrated amplification or polyploidy (p<0.001).

Table 4.3 Results of FISH analysis with HercepTest™ score.

| HercepTest™ score | FISH analysis | | |
	no amplification	borderline amplification/ polyploidy	amplification
0	n=7		
1+	n=12		
2+	n=4	n=1	
3+			n=4

p<0.001

Relationships with microvessel counts:

Membranous EGFR expression was inversely correlated with a microvessel count > median (p=0.05). Both overall and membranous EGFR expression were inversely associated with a microvessel count in the upper tertile (both p=0.05). There were no significant relationships between p53, Bcl-2, c-erbB-2 or p53+/EGFR+ expression and microvessel counts (table 4.4).

Multivariate analysis:

Cox proportional hazards regression analysis was used to define biological markers with independent predictive value with respect to cancer-specific survival (table 4.5). The most significant independent prognostic factor was nodal status. The addition of Bcl-2 immunointensity produced a statistically significant improvement in the model.

Figure 4.3 c-erbB-2 fluorescence in-situ hybridisation analysis.

a) c-erbB-2 gene amplification. The red signals represent copies of the c-erbB-2 gene and the green signals represent copies of the centromere of chromosome 17. The nuclei contain clusters of red signals indicating prominent c-erbB-2 gene amplification. The HercepTest™ score of this specimen was 3+.
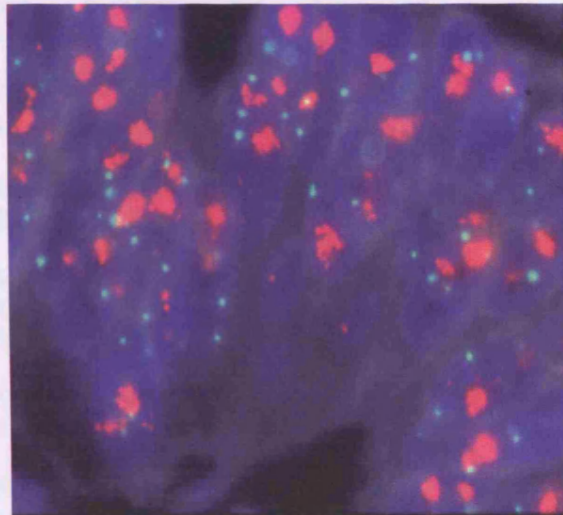
b) borderline c-erbB-2 gene amplification and polyploidy. The HercepTest™ score of this specimen was 2+.
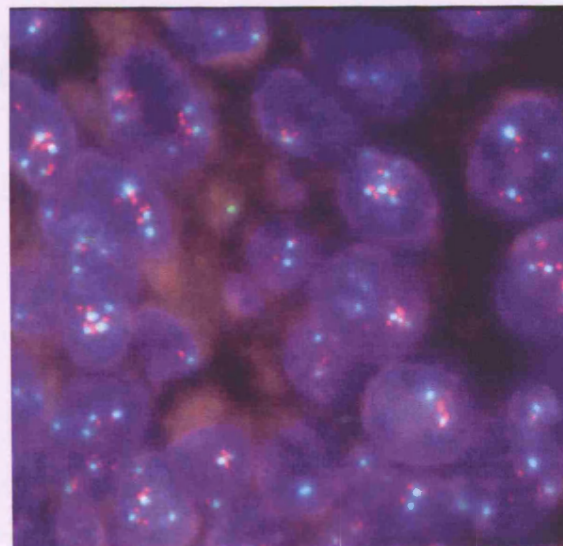
Table 4.4 Relationships between Chalkley counts and p53, Bcl-2, and EGFR immunoexpression (n=180).

prognostic factor	Chalkley count			Chalkley count		
	< median	> median	χ^2	lower tertiles	upper tertile	χ^2
nuclear p53-						
negative	53	49		63	39	
positive	41	37	p=0.94	51	27	p=0.62
Bcl-2-						
negative	64	52		76	40	
positive	29	32	p=0.33	36	25	p=0.39
negative	64	52		76	40	
weak	21	21	p=0.53	26	16	p=0.55
strong	8	11		10	9	
EGFR-						
negative	39	45		47	37	
positive	55	41	p=0.14	67	29	p=0.05^{inverse}
other	58	65		72	51	
membranous	36	21	p=0.05^{inverse}	42	15	p=0.05^{inverse}

Table 4.5 Multivariate analysis.

factor	Hazard ratio	95% confidence interval	p-value
nodal status-			
0-	1.00		0.0001
1-	1.70	1.06-2.72	
2-	3.11	1.88-5.15	
Bcl-2 expression-			
negative-	1.00		0.02
weak-	0.49	0.28-0.84	
strong-	1.24	0.64-2.37	
grade-			
well/moderate-	1.00		0.15
poor-	1.34	0.90-2.00	

Discussion.

In this study immunoreactivity for Bcl-2 was associated with a favourable outcome in NSCLC. This finding is in agreement with several studies (Pezzella F et al, 1993)(Walker C et al, 1995)(Fontanini G et al, 1995b)(Apolinario RM et al, 1997)(Fontanini G et al, 1998b)(Laudanski J et al, 1999) whilst others have not demonstrated a survival advantage (Anton RC et al, 1997)(Fleming MV et al, 1998). These variations in results may be in part due to inconsistent or ill-defined criteria for Bcl-2 positivity. In this regard a subset of strongly immunopositive cases was observed with a significantly worse prognosis compared to the more weakly staining cases. The outcome for strongly Bcl-2 staining cases was similar to those that showed no immunoreactivity. Bcl-2 immunointensity was shown to be an independent prognostic factor. To my knowledge, no other study has shown the intensity of Bcl-2 immunoreactivity to have any influence on outcome.

Weak Bcl-2 staining is present in the basal cells of normal bronchial epithelium (Walker C et al, 1995)(Koukourakis MI et al, 1997b). A study of dysplastic bronchial epithelium has shown increasing Bcl-2 expression as the dysplasia becomes more severe associated with loss of basal cell staining whilst Bcl-2 expression is reduced in overt NSCLC (Walker C et al, 1995). A similar finding has been demonstrated in cervical intraepithelial neoplasia (CIN) where Bcl-2 immunoexpression is more frequently seen in CIN-3 than CIN-1 and -2 lesions (Saegusa M et al, 1995) with reduced expression found in cervical squamous cell carcinoma (Dobbs SP et al, 1998). These observations suggest that the loss of Bcl-2 expression could be a late event in tumorigenesis, occurring as the malignant process evolves (Walker C et al, 1995)(Saegusa M et al, 1995)(Koukourakis MI et al, 1997b)(Dobbs SP et al, 1998). This is in keeping with the finding that the majority of tumours in this study were Bcl-2 negative in contrast to the high levels of expression in dysplasia reported in previous studies. The weakly staining tumours, which have a good prognosis, show an immunointensity similar to that of basal respiratory epithelial cells raising the possibility that these tumours have not lost their inherent Bcl-2 expression. Loss of Bcl-2 expression may indicate more severe molecular dedifferentiation resulting in a more aggressive phenotype.

In contrast, the strongly staining cases, which have a significantly worse prognosis compared to weakly staining cases, may represent Bcl-2 amplification or up-regulation. This may be in response to other tumour-related poor prognostic markers; for instance c-myc regulated proliferation (Evan GI et al, 1992) or hypoxia (Kobayashi N et al, 1999). Furthermore Bcl-2 transfection has been shown to enhance malignant transformation and to induce the expression

of transcription activator protein-1 (AP-1) (Amstad PA et al, 1997). Tumours with strong Bcl-2 immunostaining may therefore activate this pathway facilitating tumour growth and invasion.

There was no correlation between p53 and Bcl-2 expression in this study, a finding consistent with other studies (Walker C et al, 1995)(Koukourakis MI et al, 1997b)(Apolinario RM et al, 1997). However some studies have found an inverse relationship (Pezzella F et al, 1993)(Fontanini G et al, 1995b). A previous study has shown apoptosis occurring independently of Bcl-2 and p53 expression in NSCLC (O'Neill AJ et al, 1996). This suggests the effects of Bcl-2 and p53 may be altered by other oncogene products or regulators of apoptosis.

Previous studies have shown Bcl-2 expression to be inversely related to angiogenesis which may account for the favourable prognosis in immunopositive patients (Koukourakis MI et al, 1997b)(Fontanini G et al, 1998b). There was no correlation between Bcl-2 expression and microvessel count in this study and so this does not explain the prognostic findings in this series.

Figure 4.4 Proposed pathway for Bcl-2 expression in NSCLC.
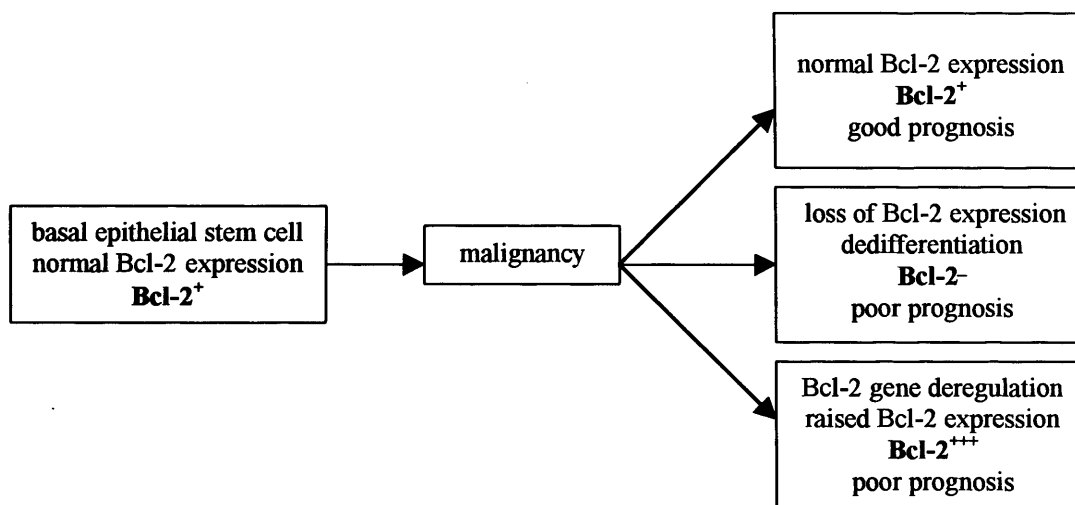
In agreement with other studies (McLaren R et al, 1992)(Nishio M et al, 1996) this study did not find p53 immunoexpression to be of prognostic influence in this patient series. Loss of wild-type p53 expression leads to angiogenesis by decreasing thrombospondin-1 expression (Dameron K et al, 1994). Wild-type p53 decreases VEGF expression (Mukhopadhyay D et al, 1995) whilst mutant p53 increases protein kinase C induced VEGF expression (Keiser A et al, 1994). Immunoexpression of mutant p53 would therefore be expected to correlate with angiogenesis. We found no relationship between p53 expression and microvessel counts. This result is in agreement with previous studies (Giatromanolaki A et al, 1996a)(Giatromanolaki A

et al, 1998b) but not all (Fontanini G et al, 1997c). It is possible that any regulating role of p53 or p21 on angiogenesis may be in tandem with other oncogenes e.g. erb receptors (Giatromanolaki A et al, 1996b). A recent study demonstrated NSCLC tumours that were both p53 and EGFR negative were associated with improved survival (Ohsaki Y et al, 2000). In our study cases that expressed both p53 and EGFR had a non-significant trend towards a poor outcome.

Although neither membranous nor overall EGFR expression was associated with prognosis in this study, there was an inverse relationship with microvessel count. This result is in keeping with a previous study in NSCLC showing erb receptor expression to be inversely related to angiogenesis and erb expressing tumours with low vascularity to have a similarly poor outcome as highly vascular tumours (Giatromanolaki A et al, 1996b). These *in vivo* findings are in contrast to *in vitro* studies where the erb receptors activation is thought to be pro-angiogenic by upregulating VEGF, IL-8 and bFGF expression (Tsai J-C et al, 1995)(Bruns CJ et al, 2000). Our results suggest NSCLC tumours may not need to stimulate intense angiogenesis in the presence of EGFR amplification. Angiogenesis and the erb-receptor pathways may potentially be separate mechanisms for tumour invasion and metastasis in NSCLC.

This study demonstrated membranous c-erbB-2 overexpression using the HercepTest™ in only 10/298 (3.4%) NSCLC resections, in agreement with a previous study (Pastorino U et al, 1997) but a lower proportion than in other studies (Kern JA et al, 1990). C-erbB-2 overexpression was found in 5/46 (10.9%) biopsy specimens from cases with stage IV disease. Apart from occasional single tumour cells, cytoplasmic c-erbB-2 expression was not seen. This is in contrast to other studies employing different antibodies and techniques (Weiner DB et al, 1990)(Tateishi M et al, 1991)(Harpole DH et al, 1995a)(Giatromanolaki A et al, 1996a)(Fontanini G et al, 1998a)(Koukourakis MI et al, 1999). Higher stage NSCLC, especially stage IV disease, were more frequently HercepTest™ positive. This suggests that alterations in c-erbB-2 gene expression may be a late event in NSCLC.

The c-erbB-2 status of a tumour may be of potential clinical importance. HerceptTest™ positive (2+ or 3+) breast tumours may respond to treatment with the novel anti-c-erB-2/HER2/neu antibody Herceptin™ (Cobleigh MA et al, 1999). Furthermore combination of standard cytotoxic chemotherapy with Herceptin™ results in prolonged time to tumour progression and overall survival in patients with metastatic breast carcinoma (Baselga J et al, 1998)(Slamon D et al, 1998). Excellent interlaboratory agreement for c-erbB-2 immunoreactivity in breast tumours using the polyclonal antibody in the HercepTest™ (Dako reference number A0485) but different assay methods and scoring systems has recently been demonstrated (Jacobs TW et al, 2000).

We demonstrated high specificity for the HercepTest™ with reliable, reproducible staining in NSCLC clearly identifying 2+ and 3+ cases when analysed independently in separate centres. However there are some questions over the sensitivity of the HercepTest™ in breast cancer with up to 60% showing c-erbB-2 overexpression (Roche PC et al, 1999) in contrast to 20-30% generally found with CB11 and 4D5 monoclonal antibodies (Allred DC et al, 2000).

All the cases tested that scored 3+ in the HercepTest™ showed c-erbB-2 gene amplification on FISH analysis. One of the 5 cases tested that scored 2+ demonstrated areas of borderline gene amplification and areas of polyploidy. All the HercepTest™ negative cases tested (0 or 1+) showed no c-erbB-2 gene amplification or polyploidy. Cases scoring 3+ with the HercepTest™ appear to reliably detect c-erbB-2 gene amplification. This finding is supported by studies in breast cancer (Graziano C, 1998). The low frequency of c-erbB-2 gene amplification in NSCLC demonstrated in our study is similar to the results from another study in which only one out of 17 tumours with c-erbB-2 immunoexpression was found to have c-erbB-2 gene amplification (Harpole DH et al, 1995b). Several studies on NSCLC have demonstrated cytoplasmic immunostaining for c-erbB-2 in up to 60% of tumours and to be associated with poor outcome. In view of the results from FISH analysis, these study results may reflect staining of c-erbB-2 protein at normal levels of gene expression. Cytoplasmic c-erbB-2 immunostaining may represent internalisation of the receptor and this may be associated with alterations in intracellular signalling.

The results indicate that unlike operable breast cancer (Wright C et al, 1989)(Graziano C, 1998), gene amplification and strong membranous c-erbB-2 protein over-expression are uncommon in resected NSCLC tumours. As the monoclonal antibody appears to be an effective treatment only in this subgroup of patients, Herceptin™ would target a small proportion of stage I-IIIa disease and therefore be of limited clinical use in the adjuvant setting for NSCLC. Gene amplification was generally found in the more advanced tumours and there may be a potential role for Herceptin™ in metastatic NSCLC. The finding that c-erbB-2 over-expression is more common in higher stage NSCLC is in agreement with previous studies demonstrating an association between c-erbB-2 expression and poor prognosis (Weiner DB et al, 1990)(Tateishi M et al, 1991)(Harpole DH et al, 1995a).

In conclusion the prognostic role of Bcl-2 immunoreactivity in NSCLC remains controversial. This study found that strongly staining tumours had a worse outcome than tumours that stained weakly. Strongly staining cases may represent amplification or up-regulation whereas weak staining may represent normal levels of Bcl-2 expression. Further studies should emphasise the different intensity and localisation of staining as this may account for the disparate results in

prognosis from previous studies. Studies of c-erbB-2 expression have used different monoclonal or polyclonal antibodies directed toward different epitopes of the receptor. Standardisation of c-erbB-2 immunohistochemical analysis is required before incorporation into routine clinical practice. The HercepTest™ in NSCLC appears to be specific for gene amplification in cases with a 3+ score and reproducible in different centres. The HercepTest™ demonstrated immunopositivity in only a small proportion of NSCLC tumours suggesting that the clinical use of c-erbB-2 antibodies in the treatment of this disease may be limited, especially in the adjuvant setting.

Chapter 5

The Gelatinase Family in
Operable Non-Small Cell Lung Cancer
and the Development of a
Molecular Sub-Staging Prognostic Model

The Gelatinase Family in Operable Non-Small Cell Lung Cancer and the Development of a Molecular Sub-Staging Prognostic Model.

Introduction.

Malignant cells either secrete proteases or acquire protease activity from host stromal cells or inflammatory cells to allow them to break through collagenous protein barriers. Matrix metalloproteinase (MMP)-2 (gelatinase A) and MMP-9 (gelatinase B) degrade collagen type IV, the major component of the basement membrane. MT1-MMP is a membrane-bound MMP that specifically activates MMP-2 (He C et al, 1989). Over-expression of MMPs facilitates metastatic spread of disease. MMP levels relate to invasive and metastatic potential *in vitro* (Kawamata H et al, 1995) whilst synthetic MMP inhibitors decrease the spread of cancer *in vivo* (Talbot DC et al, 1996).

MMP-2 and MMP-9 have been demonstrated in the tumour, stromal and inflammatory cells of many types of cancer including breast, gastric and lung carcinoma (Basset P et al, 1990)(Sato H et al, 1992)(Urbanski SJ et al, 1992)(Brown PD et al, 1993a)(Nomura H et al, 1995)(Tomita T et al, 1996). The level of MMP-2 has been associated with the invasive behaviour and metastatic potential of most histological types of lung neoplasm (Brown PD et al, 1993a). Over-expression of MMP-2 has been found to be associated with advanced stage (Thomas P et al, 2000). Increased expression of MMP-9 mRNA and MMP-9 protein have been demonstrated in NSCLC (Muller D et al, 1991)(Brown PD et al, 1993)(Nagakawa H et al, 1994)(Kodate M et al, 1997). MMP-9 mRNA is present in the stroma closely surrounding tumour cell clusters (Collier IE et al, 1988) and in the tumour cells of squamous cell carcinoma (Cannette-Soler R et al, 1994) and adenocarcinoma (Urbanski SJ et al, 1992). Higher levels of MMP-9 mRNA have been found in stage III NSCLC compared to stages I and II (Brown PD et al, 1993a). The expression of either MMP-9 or MMP-2 has been shown to confer a worse prognosis in early stage adenocarcinoma of the lung (Kodate M et al, 1997). The expression of MMP-2 in NSCLC can frequently be super-imposed on to that of MT1-MMP (Urbanski SJ et al, 1992)(Tokaraku M et al, 1995). MT1-MMP has been shown to be expressed more frequently in stage III NSCLC than in stages I and II and MT1-MMP mRNA levels to correlate with the presence of lymph node disease (Tokaraku M et al, 1995).

The extracellular activity of MMPs is in part controlled by naturally occurring tissue inhibitors of metalloproteinases (TIMPs). TIMP-1 forms a complex with proMMP-9 blocking its activation (Goldberg GI et al, 1992). TIMP-2 forms a complex with MMP-2 and its C-terminal strongly binds proMMP-2 (Howard EW et al, 1991). TIMP-1 and TIMP-2 are present in both

tumour and non-neoplastic lung (Urbanski SJ et al, 1992)(Nagakawa H et al, 1994). Studies have found TIMP-1 over-expression in advanced disease (Thomas P et al, 2000) and high TIMP-1 mRNA levels to be associated with a poor prognosis (Fong KM et al, 1996). This is at odds with the inhibitory action on invasion demonstrated by TIMPs *in vitro* studies. Similarly a study has demonstrated increased TIMP-2 expression to occur in NSCLC tumours with nodal metastases (Karameris A et al, 1997).

Currently the best prognostic index for operable NSCLC is the TNM staging system. Molecular biology holds the promise of predicting outcome for the individual patient and identifying novel therapeutic targets. Angiogenesis, the apoptosis regulatory factors p53 and Bcl-2, MMP-2, MMP-9 and the erb/HER type I tyrosine kinase receptors are all implicated in the pathogenesis of NSCLC. The aim of this study is to evaluate the relationship between MMP-2, MMP-9, MT1-MMP, TIMP-1 and TIMP-2 expression in NSCLC and to assess the impact of expression on clinicopathological parameters and survival. Using the results from all these studies the aim was to develop a model to predict the outcome for the individual patient based on the molecular biology of the tumour independent of the TNM stage.

Methods.

This was a retrospective study of a series of resected stage I-IIIa NSCLC with a post-operative survival of > 60 days. Paraffin-embedded sections from the tumour periphery were stained using standard ABC immunohistochemistry, as per methods section, for MMP-2, MMP-9 and TIMP-2. Results for microvessel counts and p53, Bcl-2, EGFR and c-erbB-2 expression were taken from the studies described in previous chapters.

Evaluation of immunohistochemistry:
The extent and pattern of reactivity for each antibody was recorded by two observers. The extent of expression was scored 0 for no staining, <20%, 20-50% and 50-100%. A similar semi-quantitative scale of 0, +, ++ or +++ was used to assess the intensity of staining in comparison to a known positive control. This scoring system was applied to tumour cell and stromal reactivity for MMP-2, MMP-9 and TIMP-2. Cases were called positive if staining intensity was ++ or +++ over at least 20% of the tumour. Sections were analysed in a blinded fashion and the results of the immunohistochemistry, tumour status and outcome correlated subsequently.

Statistics:
Statistical analysis was performed using the SPSS software system (SPSS for Windows Version 9.0). The Chi-squared test was used to analyse the associations between categorical

clinicopathological variables. Cancer-specific survival curves were plotted using the Kaplan-Meier method and the log-rank test was used to assess the statistical significance of differences between groups. The joint effects of covariables that were significant at the 0.25 level in univariate analysis were further examined via Cox regression using a forward selection procedure. The 0.05 level of significance was used for entering or removing a covariable from this model.

Results.

Specificity of immunoreactivity:

During the optimisation of the immunohistochemical technique a positive control tissue was included for which the pattern of reactivity with each antibody was known. Evaluation of this section with each immunohistochemistry run ensured that the appropriate staining pattern was achieved. All negative controls were negative.

MMP-2 expression:

MMP-2 immunostaining was performed in 169 cases. MMP-2 immunoexpression was observed in stromal fibroblasts, inflammatory cells and the cytoplasm of tumour cells. No case demonstrated convincing tumour cell membrane staining. Staining was usually more intense where tumour cells were in close proximity to the stroma (figures 5.1a & b). MMP-2 tumour cell expression was seen in 41/169 (24.3%) of cases and was not associated with outcome (p=0.95) (figure 5.2a). Stromal MMP-2 expression was seen in 103/169 (60.9%) of cases and was not of prognostic significance (p=0.12)(figure 5.2b). Stromal staining was seen more frequently in squamous cell carcinomas (p<0.001). Stromal and tumour cell MMP-2 were frequently co-expressed (p=0.010). There were no other associations with clinicopathological findings for either tumour cell or stromal MMP-2 expression.

MMP-9 expression:

MMP-9 immunostaining was performed in 169 cases. Reactivity for MMP-9 was observed in stromal fibroblasts and infiltrating macrophages and localised to the cytoplasm of tumour cells. MMP-9 expression was frequently more intense at the infiltrating edge of the tumour (figures 5.1c, d & e). MMP-9 tumour cell expression was recorded in 88/169 (52%) of cases and conferred a poor prognosis (p=0.001) (figure 5.2c). MMP-9 tumour cell expression was associated with poor outcome in stage II disease (p=0.02) but did not reach significance in either stage I (p=0.06) or stage IIIa disease (p=0.87). MMP-9 stromal expression was seen in 79/169 (47%) of cases and was not prognostic (p=0.99)(figure 5.2d). Stromal staining was found more frequently in large cell and squamous cell carcinoma histological sub-types

(p=0.003). Stromal and tumour cell MMP-9 were frequently co-expressed (p=0.015). There were no other associations with clinicopathological findings for either tumour cell or stromal MMP-9 expression.

TIMP-2 expression:

TIMP-2 immunostaining was performed in 157 cases. TIMP-2 immunoreactivity was observed in stromal fibroblasts and tumour cell cytoplasm. Again no convincing membrane reactvity was observed (figures 5.1f & g). TIMP-2 tumour cell expression was seen in 68/157 (43.3%) of cases and was not associated with outcome (p=0.62) (figure 5.2e). Stromal TIMP-2 expression was seen in 123/157 (78.3%) of cases and was not of prognostic significance (p=0.68)(figure 5.2f). Stromal staining was seen more frequently in squamous cell carcinomas (p<0.001) and in males (p=0.03). There was no correlation between stromal and tumour cell TIMP-2 expression (p=0.61). There were no other associations with clinicopathological findings for either tumour cell or stromal MMP-2 expression.

MT1-MMP and TIMP-1 expression:

A range of different antibodies, antibody concentrations and antigen retrieval techniques were tested for both MT1-MMP and TIMP-1 on the formalin-fixed tissue but no convincing staining was obtained under any conditions and therefore these antigens were not evaluated.

Relationships between MMP-2, MMP-9 and TIMP-2 expression:

There was significant co-expression of MMP-2 and MMP-9 in the tumour cells (p=0.001) and in the stroma (p=0.006). There was no association between TIMP-2 tumour cell immunostaining and MMP-2 or MMP-9 tumour cell staining (p=0.06 and p=0.39 respectively). Immunoreactivity for TIMP-2 in the stroma correlated with stromal MMP-2 (p=0.001) and MMP-9 (p=0.004) positivity.

Relationship between MMP-2, MMP-9, TIMP-2 and EGFR expression:

Tumour cell co-expression of MMP-9 and EGFR was found in 62/169 (37%) cases (p<0.0001). Membranous EGFR and tumour cell MMP-9 were also co-expresssed in 38/169 (22%) cases (p=0.002). There was no association between cytoplasmic EGFR and tumour cell MMP-9 expression (p=0.18). Tumour cell co-expression of EGFR and MMP-9 were associated with a poor outcome (p=0.0001)(figure 5.2g) as was co-expression of membranous EGFR and MMP-9 (p=0.003)(figure 5.2h). EGFR and MMP-9 tumour cell co-expression was associated with poor outcome in stage I disease (p=0.001) but did not reach significance in either stage II (p=0.13) or stage IIIa disease (p=0.74). Cytoplasmic EGFR and tumour cell MMP-9 co-expression was not

Figure 5.1 Immunohistochemistry for MMP-2, MMP-9 and TIMP-2 tumour cell and stromal staining.

a) MMP-2 tumour cell staining
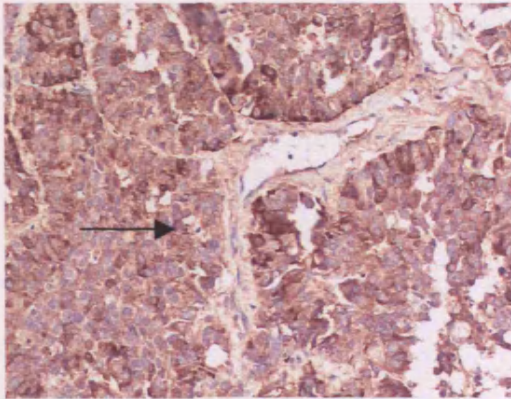
b) MMP-2 stromal cell staining
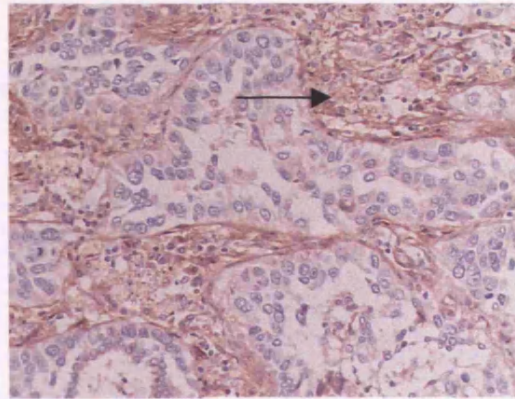
c) MMP-9 tumour cell staining
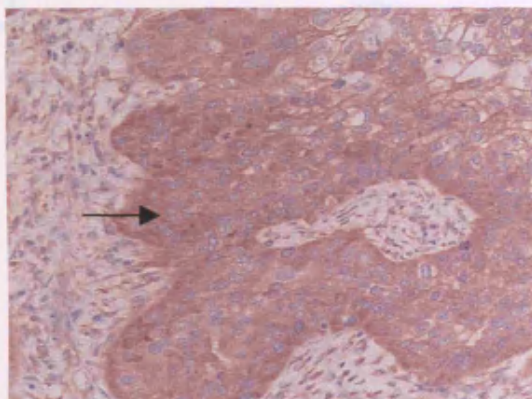
d) MMP-9 stromal cell staining
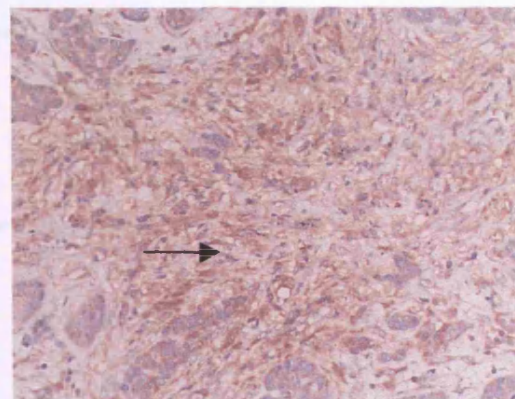
e) MMP-9 staining inflammatory cell infiltrate
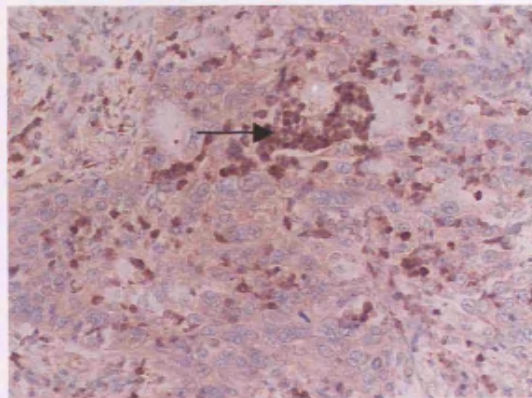
f) TIMP-2 tumour cell staining
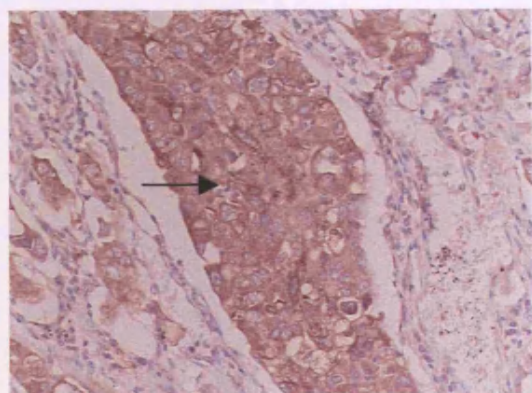
g) TIMP-2 stromal cell staining
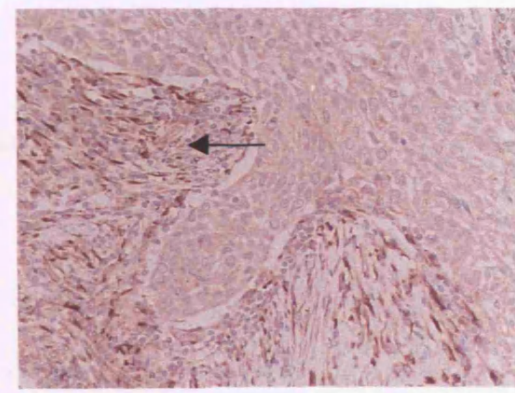
Figure 5.2 Survival curves for MMP-2, MMP-9 and TIMP-2 tumour cell and stromal expression and overall and membranous EGFR/tumour cell MMP-9 co-expression.

figure 5.2a:

MMP-2 tumour cell expression

p=0.95

figure 5.2b:

MMP-2 stromal expression

p=0.12

figure 5.2c:

MMP-9 tumour cell expression

p=0.001

figure 5.2d:

MMP-9 stromal expression

p=0.99
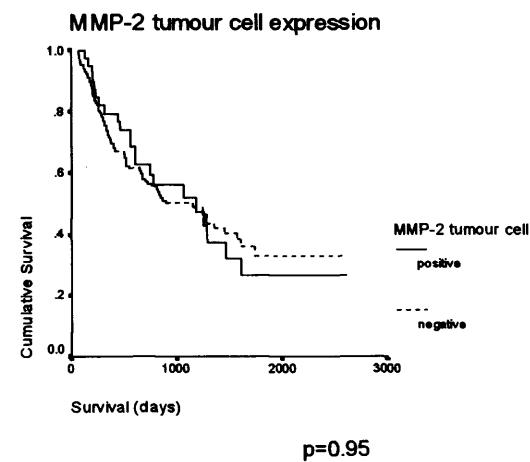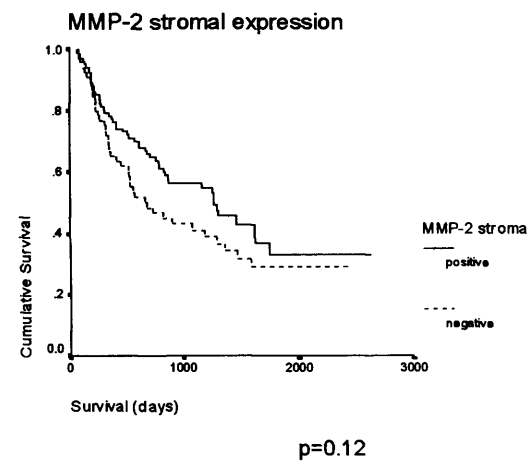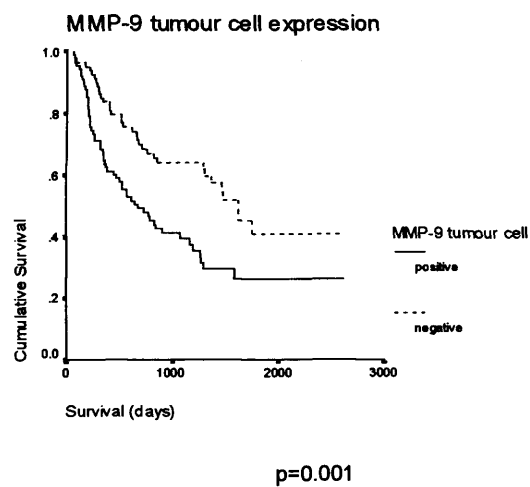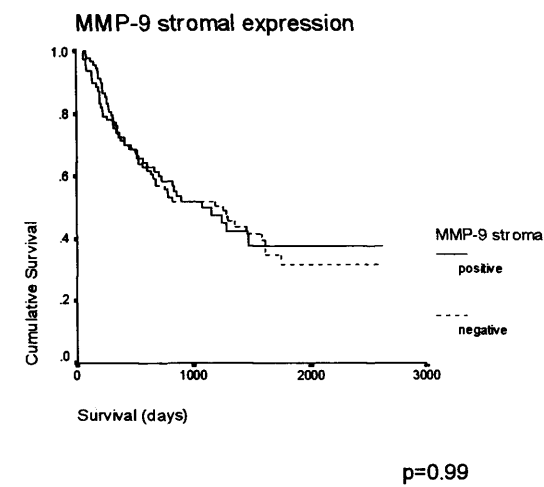
Figure 5.2 (continued).

figure 5.2e:

TIMP-2 tumour cell expression

p=0.62

figure 5.2f:

TIMP-2 stromal expression

p=0.68

figure 5.2g:

EGFR and MMP-9 tumour cell co-expression

p=0.0001

figure 5.2h:

Membranous EGFR and MMP-9 tumour cell co-expression

p=0.003
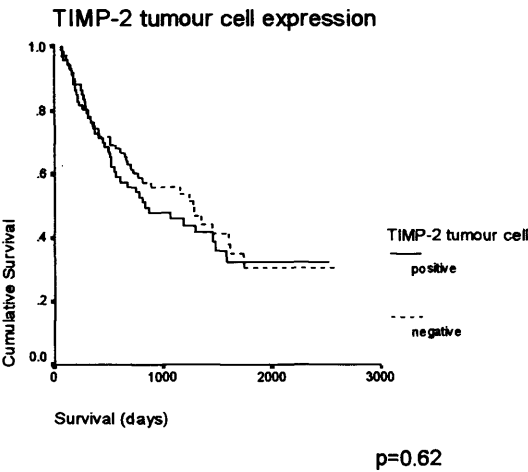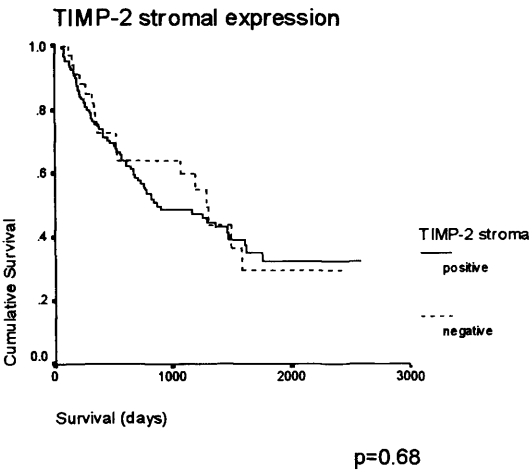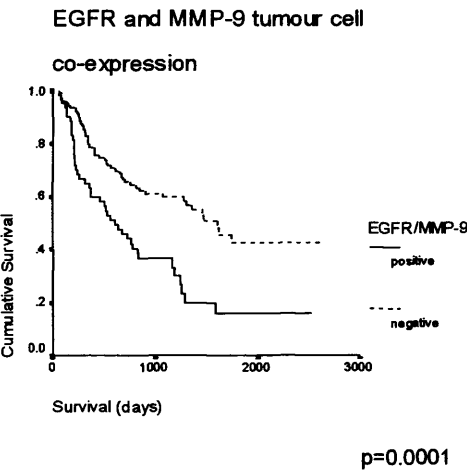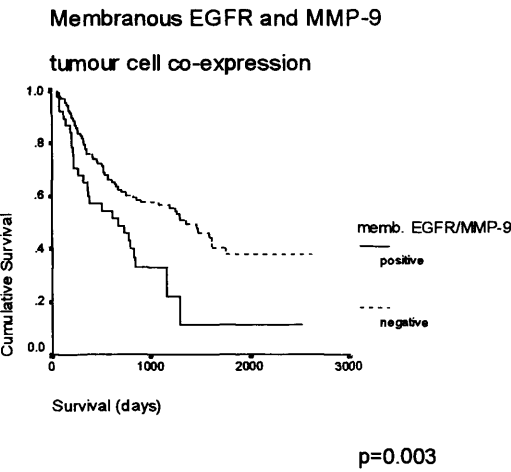
Table 5.1 Multivariate analysis of univariate factors with p<0.25. The use of EGFR/MMP-9 tumour cell expression increased the prognostic significance of Bcl-2 and Chalkley count.

Table 5.1a MMP-9 and EGFR as separate variables.

Factor	HR	CI	p-value
tumour cell MMP-9 expression-			
negative-	1.00		0.003
positive-	1.94	1.25-3.04	
Bcl-2 expression-			
negative-	1.00		0.01
positive-	0.53	0.33-0.86	
Chalkley count-			
< median-	1.00		0.02
> median-	1.70	1.09-2.66	
stage-			
stage I-	1.00		0.02
stage II-	1.75	1.05-2.93	
stage IIIa-	1.95	1.19-3.42	

Table 5.1b: MMP-9 and EGFR as a combined variable.

Factor	HR	CI	p-value
tumour cell EGFR/MMP-9 expression-			
negative-	1.00		0.0001
positive-	2.40	1.54-3.72	
Bcl-2-			
negative-	1.00		0.005
positive-	0.50	0.31-0.81	
Chalkley count-			
< median-	1.00		0.007
> median-	1.84	1.18-2.89	
stage-			
stage I-	1.00		0.02
stage II-	1.69	1.01-2.81	
stage IIIa-	1.95	1.15-3.29	

Figure 5.3 Survival curves incorporating microvessel count, MMP-9 tumour cell expression and Bcl-2 expression.

figure 5.3a:

Chalkley count and MMP-9 tumour cell expression

p=0.0002

figure 5.3b

Chalkley count < median and MMP-9 tumour cell negative

p=0.07

figure 5.3c:

Chalkley count > median or MMP-9 tumour cell positive

p=0.28

figure 5.3d:

Chalkley count > median and MMP-9 tumour cell positive

p=0.06
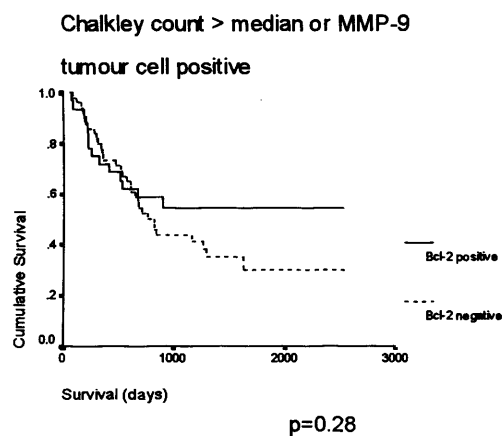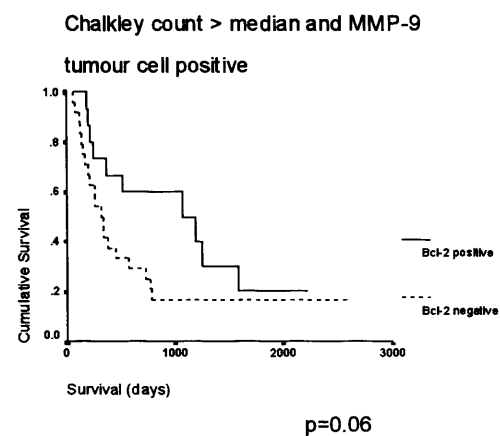
legend for figure 5.3a:

A Chalkley count < median, MMP-9 tumour –ve   13 cancer deaths/40 (33%)
B Chalkley count > median or MMP-9 tumour +ve   46 cancer deaths/85 (54%)
C Chalkley count > median, MMP-9 tumour +ve   30 cancer deaths/41 (73%)

A vs B   p=0.02   A vs C   p<0.0001   B vs C   p=0.02

legend for figures 5.3b, 5.3c, 5.3d:

Chalkley count < median, MMP-9 tumour –ve, Bcl-2 +ve   1 cancer death   / 10   (10%)
Chalkley count < median, MMP-9 tumour –ve, Bcl-2 –ve   12 cancer deaths / 30   (40%)

Chalkley count > median or MMP-9 tumour +ve, Bcl-2 +ve   14 cancer deaths / 33   (42%)
Chalkley count > median or MMP-9 tumour +ve, Bcl-2 -ve   32 cancer deaths / 52   (62%)

Chalkley count > median, MMP-9 tumour +ve, Bcl-2 +ve   10 cancer deaths / 16   (63%)
Chalkley count > median, MMP-9 tumour +ve, Bcl-2 -ve   20 cancer deaths / 25   (80%)

Figure 5.4 Survival curves incorporating microvessel count, EGFR/MMP-9 tumour cell expression and Bcl-2 expression.

figure 5.4a:

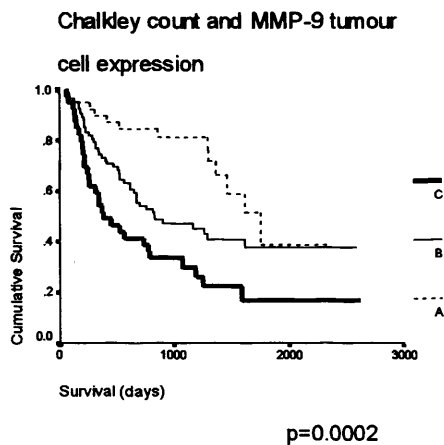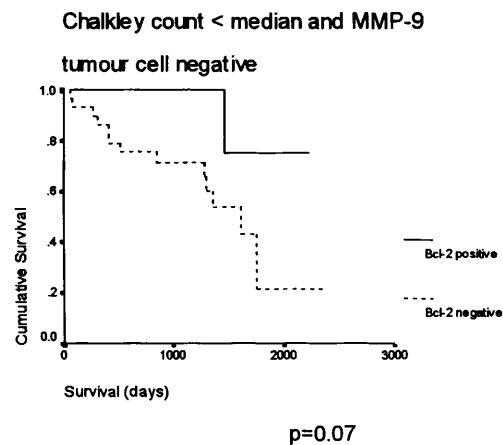**Chalkley count and EGFR/MMP-9 tumour cell expression**

p=0.0001

figure 5.4b:

Chalkley count < median and tumour cell EGFR/MMP-9 negative

p=0.10

figure 5.4c:

Chalkley count > median or tumour cell EGFR/MMP-9 expression

p=0.22

figure 5.4d:

Chalkley count > median and tumour cell EGFR/MMP-9 expression
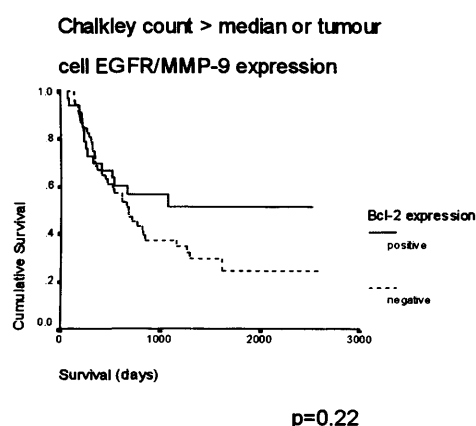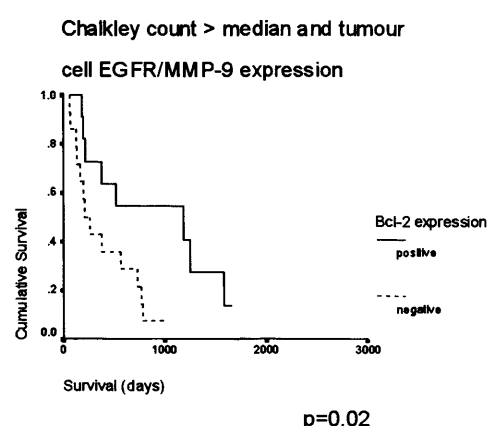
p=0.02

legend for figure 5.4a:

A Chalkley count < median, EGFR/MMP-9 –ve   17 cancer deaths/52 (33%)
B Chalkley count > median or EGFR/MMP-9 +ve   51 cancer deaths/87 (59%)
C Chalkley count > median + EGFR/MMP-9 +ve   21 cancer deaths/27 (78%)

A vs B   p=0.002   A vs C   p<0.0001   B vs C   p=0.02

legend for figures 5.4b, 5.4c, 5.4d:

Chalkley count < median, EGFR/MMP-9–ve, Bcl-2+ve   2 cancer deaths/14 (14%)
Chalkley count < median, EGFR/MMP-9–ve, Bcl-2–ve   15 cancer deaths/38 (39%)

Chalkley count > median or EGFR/MMP-9+ve, Bcl-2+ve   16 cancer deaths/35 (46%)
Chalkley count > median or EGFR/MMP-9+ve, Bcl-2–ve   35 cancer deaths/52 (67%)

Chalkley count > median, EGFR/MMP-9+ve, Bcl-2+ve   8 cancer deaths/12 (67%)
Chalkley count > median, EGFR/MMP-9+ve, Bcl-2-ve   13 cancer deaths/15 (87%)

associated with poor outcome. Tumour cell immunostaining for both TIMP-2 and EGFR was seen in 31/157 (20%) cases and were found to be inversely correlated (p=0.03). This association was not seen with membranous EGFR expression and TIMP-2 (p=0.31).

Relationships with microvessel counts:

There was no correlation between either MMP-2, MMP-9 or TIMP-2 tumour cell expression and microvessel count. There was an inverse correlation between high microvessel counts and MMP-2 (p=0.03) but not with MMP-9 (p=0.07) or TIMP-2 (p=0.82) stromal expression.

Development of molecular sub-staging model:

Cox proportional hazards regression analysis was used to define biological markers with independent predictive value with respect to cancer-specific survival (tables 5.1a & b). Tumour cell MMP-9 or MMP-9/EGFR co-expression, Bcl-2 immunoreactivity, Chalkley count > median and tumour stage were all significant independent prognostic factors. The addition of EGFR to the analysis increased the independent prognostic significance of the Chalkley count and Bcl-2 expression

A model using the molecular biological markers found to have independent prognostic significance was developed. Patients with a Chalkley count > median and tumour cell MMP-9 expression had a significantly worse prognosis compared to patients with only one or neither of these factors (figure 5.3a). The addition of Bcl-2 to the model indicated a better survival in each sub-group (figures 5.3b, c & d). Similarly using cases with both EGFR and MMP-9 tumour cell expression also produced a prognostic model (figures 5.4a, b, c & d).

Discussion.

MMPs are part of the proteolytic cascade that degrades the ECM and allows the migration of tumour and endothelial cells. In particular the gelatinases MMP-2 and MMP-9 are capable of forming gaps in the basement membrane to facilitate invasion and metastatic spread. TIMP-2 is a natural inhibitor of MMP activity, especially MMP-2 whilst TIMP-1 is the natural inhibitor of MMP-9. The use of fixed tissue affords superior tissue morphology and allows a better choice of tissue section for analysis, as well as more detailed analysis of the localisation of staining. To date, most tissue studies of MMP expression have been limited by the fact that the antibodies available have not worked reproducibly on routinely fixed paraffin-embedded tissue. Using paraffin-embedded NSCLC tissue sections, this study has demonstrated strong, specific and reproducible staining for both stromal and cytoplasmic tumour cell MMP-2, MMP-9 and TIMP-2 expression. Despite attempts, MT1-MMP and TIMP-1 did not exhibit staining on fixed tissue

sections, but it is possible as more antibodies are generated, species will become available that will allow analysis.

MMP-9 tumour cell expression was associated with a poor prognosis on univariate and multivariate analysis, especially in stage I and II disease. This provides further evidence in support of an important role for proteases in the malignant process. This contradicts a study of lung adenocarcinomas in which MMP-9 expression, stained using a polyclonal antibody, was not prognostic. However the pattern of stromal or tumour cell MMP-9 expression was not discussed (Fujise N et al, 2000).

The proteolytic activity of the gelatinases has been shown to assist the process of angiogenesis *in vitro* and *in vivo* (Schnaper HW et al, 1993)(Johnson MD et al, 1994)(Itoh T et al, 1998). Angiogenesis is inhibited by TIMP-2 (Murphy AN et al, 1993). There was no association between tumour cell MMP-2, MMP-9 and TIMP-2 expression and microvessel counts. There was a significant inverse correlation between stromal MMP-2 expression and high vessel counts and a similar trend for MMP-9 but not for TIMP-2. As TIMP-2 was co-expressed in the stroma with MMP-2 and MMP-9, the local balance between MMP and TIMP activity may affect angiogenesis.

There was a significant relationship between the presence of tumour cell MMP-9 reactivity and both membranous and overall EGFR immunopositivity. This suggests that EGFR may be involved in the specific up-regulation of MMP-9, a contention supported by *in vitro* studies in which activation of EGFR in solid tumour cell-lines resulted in up-regulation of this gelatinase (Rosenthal EL et al, 1998)(O-charoenrat P et al, 2000). The EGFR ligands EGF, amphiregulin and TGFα have been shown to induce the expression of MMPs (Unemori EN et al, 1994)(Kondapaka SB et al, 1997). Together these findings suggest the EGFR signalling pathway may contribute to the metastatic process by specifically up-regulating expression of MMP-9 and promoting tumour invasion. The precise mechanism involved is not understood, however EGFR stimulation may lead to the activation of the *ras*/MAPK/AP-1/ETS-1 pathway and the subsequent up-regulation of MMP-9 (Tanaka H et al, 1999)(Reddy KB et al, 1999)(Westermarck J et al, 1999). Alternatively EGFR may act by down-regulating E-cadherin as has been suggested in breast cancer (Jones JL et al, 1996). Down-regulation of E-cadherin in mouse skin carcinoma cells results in increased MMP-9 levels associated with increased motility *in vitro* and metastatic potential *in vivo* (Llorens A et al, 1998). A recent study has shown a high MMP-2 and -9:E-cadherin mRNA ratio is associated with an increased risk of recurrence after resection for stage I NSCLC (Herbst RS et al, 2000). Other potential EGFR/MMP-9 pathways may act PI3 kinase or PKC (Porter AC et al, 1998)(Leevers SJ et al,

1999). Conversely in my study TIMP-2 tumour cell expression was shown to inversely correlate with EGFR expression suggesting that the production of TIMPs may also be down-regulated by the EGFR signal pathway.

The co-expression of EGFR and MMP-9 in tumour cells was strongly associated with a poor prognosis. Up-regulation of MMP-9 in the tumour may promote invasion and metastasis and account for this finding. This does not explain why tumours with strong stromal expression for MMP-9 or those expressing MMP-2 do not appear to have a poor outcome. An alternate explanation could be that tumour cell MMP-9 staining is a surrogate marker of another substance that may also be up-regulated through the EGFR pathway.

The goal of molecular biological staging is to find a model that will predict the long-term prognosis for a patient with NSCLC on an individual basis. This will allow management decisions to be dictated by the aggressiveness of the tumour rather than the histopathological findings. Treatment may be tailored to specific metastatic pathways using novel therapeutic agents.

Multivariate analysis confirmed MMP-9 tumour cell expression, Bcl-2 expression and a Chalkley count > median to be independent molecular biological prognostic markers. To create a model for molecular sub-staging the survival curves for MMP-9 and Chalkley count > median for the presence of neither, one or both of these variables were plotted. Cases with both a raised microvessel count and MMP-9 expression had a significantly worse outcome compared to cases where only one or neither of these patterns was demonstrated. The addition of Bcl-2 expression to this model showed increased survival in each sub-group. Using this model, the presence of the good prognostic indices was associated with a cancer-related death rate of only 10% whereas the presence of a microvessel count >median, tumour cell MMP-9 expression and an absence of Bcl-2 expression conferred a cancer-related death rate of 80%.

When a case demonstrated both EGFR and MMP-9 tumour cell expression, survival was significantly decreased. Thirteen out of 15 cases with a Chalkley count > median, EGFR/MMP-9 tumour cell expression and the absence of Bcl-2 expression died from a recurrence of their cancer. As one of these died at day 61 with a post-operative empyema, only 1 out of 15 cases (6.6%) was alive and disease-free at 35 months.

This prognostic model uses only four immunohistochemical markers, all of which work on routinely processed tissue, and could easily be performed in a general pathology laboratory. The predictive value of this model will need to be validated by a prospective study and if confirmed

will provide prognostic information offering the potential for individualising patient treatment. In the future it may be possible to add the results of MT1-MMP and TIMP-1 immunoexpression into the model, both of which may be expected to influence the activity of the gelatinase system. Recent work developing anti-angiogenesis agents such as angiostatin (O'Reilly MS et al, 1994) and endostatin (O'Reilly MS et al, 1997), matrix metalloproteinase inhibitors (Rasmussen HS et al, 1997) and EGFR-targeted therapies (Cardiello F et al, 1999)(Lei W et al, 1999)(Bunn PA et al, 2000) could allow specific inhibition each of these pathways, either alone or in conjunction with traditional cytotoxic therapies.

Chapter 6

The Expression and Regulation of the Gelatinase Family in Non-Small Cell Lung Cancer Cell-Lines

The Expression and Regulation of the Gelatinase Family in
Non-Small Cell Lung Cancer Cell-Lines.

Introduction.

The expression of MMPs has been associated with tumour cell invasion and metastasis due to the ability of these proteinases to hydrolyze the extracellular matrix. MMP-2 and -9 (gelatinase A and B) are thought to play an important role in invasion as they degrade collagen type IV, the major component of the basement membrane. MMP-9 is an inducible metalloproteinase unlike the constitutively expressed MMP-2 (Westermarck J et al, 1999). MT1-MMP is a membrane bound member of the gelatinase family closely involved in the activation of proMMP-2 (He C et al, 1989). TIMP-1 and TIMP-2 are naturally occurring endogenous inhibitors of all MMPs that bind to the zinc binding site of active MMPs. TIMP-1 has a special affinity for proMMP-9 whilst TIMP-2 preferentially binds pro and active MMP-2 (Howard EW et al, 1991)(Goldberg GI et al, 1992). Together MMP-2, MMP-9, MT1-MMP, TIMP-1 and TIMP-2 make up the gelatinase family.

Whilst the mechanisms regulating MMP-9 expression in NSCLC are not fully understood, previous studies have shown MMP-9 expression may be induced by a variety of stimuli including growth factors, cytokines and mutagens in various tumour cell types (Khokha R et al, 1989)(Mann JS et al, 1991)(Birkedal-Hansen H et al, 1993)(Leco KJ et al, 1994)(Benbow U et al, 1997). More recently the EGFR ligands EGF and amphiregulin have been shown to up-regulate the expression of MMP-9 but not MMP-2 in breast cancer cell lines (Kondapaka SB et al, 1997). Another study has shown similar findings in head and neck squamous carcinoma cells (O-charoenrat P et al, 2000a). This study also showed that, in EGFR-overexpressing cell-lines, proliferation was stimulated by EGF in the picomolar range and was inhibited in the nanomolar range. This biphasic growth response has been demonstrated previously (Modjtahedi H et al, 1993). The concentration of EGF-like ligands required to up-regulate MMP-9 expression and enhance invasion was at least a log higher (in nM range) than that required to induce proliferation in EGFR-over-expressing head and neck squamous cell-lines (O-charoenrat P et al, 2000a). A strong correlation between EGFR levels and MMP-9 mRNA and enzymatic activity and an inverse correlation with TIMP-1 mRNA has also been demonstrated in head and neck tumour cell-lines (O-charoenrat P et al, 2000b). EGF signalling blocked by *ras* and PI3 kinase inhibitors prevents EGF-induced proliferation and partly reduces EGF-induced MMP-9 secretion in breast epithelial cells (Reddy KB et al, 1999). Keratinocyte migration induced by EGF, TGF-α and scatter factor/hepatocyte growth factor (SF/HGF) is associated with induction

of MMP-9 (McCawley LJ et al, 1998). A study in ovarian cancer cell-lines demonstrated EGF-induced MMP-9 expression in 2 out of 4 cell-lines. EGF-induced invasion was seen in 3 out of 4 cell-lines and this invasion was reduced in presence of anti-MMP-9 antibody (Ellerbroek SM et al, 1998).

Our immunohistochemical studies have demonstrated a strong correlation between EGFR and tumour cell MMP-9 expression *in vivo* suggesting the EGFR signalling pathway may up-regulate MMP-9 production in NSCLC. The aim of this study is to evaluate the expression of all the members of the gelatinase family in 5 NSCLC cell-lines, to assess their relationship with invasion and to investigate the hypothesis that EGFR activation specifically enhances MMP-9 expression and activity.

Methods.

The following studies were performed on all 5 NSCLC cell-lines as described in the methods chapter. Immunohistochemical staining to assess the EGFR status of each cell-line was performed on paraffin-embedded cytoblocks^{TM}. Immunointensity was graded as no staining, +, ++ or +++.

mRNA extracted from HT1080 fibrosarcoma cells was used as a positive control for all RT-PCR reactions. RT-PCR for MMP-2, MMP-9, MT1-MMP, TIMP-1 and TIMP-2 was performed on the cells cultured in serum-containing media to identify baseline mRNA expression. These experiments were repeated after cells were stimulated with 100nM EGF for 48 hours. Invasion assays over a 48 hour period through a Matrigel barrier were carried out for all cell-lines and repeated with 100nM EGF stimulation.

Previous studies have shown when conditioned media is omitted from the well the cells do not exhibit invasion of the Matrigel barrier. Studies in our own laboratory with breast cancer cell-lines have demonstrated 100ng/ml EGF to be stimulatory (unpublished). Studies on NSCLC and other solid tumour cell-lines have used EGF concentrations between 5 and 500ng/ml, with similar stimulatory effects with all concentrations (Kondapaka SB et al, 1997)(Ellerbroek SM et al, 1998)(Reddy KB et al, 1999)(O-charoenrat P et al, 2000a).

Results.

Cell-line EGFR immunoexpression.

All five NSCLC-lines demonstrated immunoexpression for EGFR with variable intensity (table 6.1 and figure 6.2).

Table 6.1 NSCLC cell-line EGFR immunostaining.

NSCLC cell-line	histology	EGFR immunointensity
H460	adenocarcinoma	+
A549	adenocarcinoma	+++
MORP	adenocarcinoma	+
SK-MES	squamous cell	+++
H647	adenosquamous	++

Baseline expression of MMP-2 and –9, MT1-MMP and TIMP-1 and TIMP-2 mRNA.

MMP-2 mRNA was demonstrated by RT-PCR in 4 out of 5 cell-lines (H460, A549, SK-MES and H647). MMP-9 mRNA was not found at baseline in any of the NSCLC cell-lines. MT1-MMP, TIMP-1 and TIMP-2 mRNA were found in all cell-lines tested (figure 6.3).

EGF-regulation of MMP-2 and –9, MT1-MMP and TIMP-1 and TIMP-2.

As assessed by RT-PCR, EGF had no overt effect on the expression of MMP-2, MT1-MMP and TIMP-1 and TIMP-2 mRNA. However MMP-9 mRNA expression was induced in 4 of the 5 cell-lines (A549, MORP, SK-MES and H647) after 48 hours of incubation with 100ng/ml EGF (figures 6.1 and 6.3).

EGF-regulation of cell invasion.

Matrigel-coated invasion wells were utilised to determine the impact of EGF on NSCLC cell-line invasion. Experiments were repeated in duplicate on 3-4 occasions. Problems were observed with the membrane occasionally becoming loose from the well-insert and with incomplete or uneven formation of the Matrigel coat. There were marked variations in the invasion index between similar condition-experiments (H647 baseline range 9.7-30.6%, A549 + EGF range 11.7-38.4%). These variations may be in part due to the membrane becoming loose during the invasion assay and altering the gradient. Alternatively incomplete or uneven Matrigel-coating of the membrane may also lead to variable invasion. The mean invasion index was higher after stimulation with EGF in all the cell-lines tested, reaching statistical significance for H460 only (p=0.02)(figure 6.4).

Figure 6.1 Agarose gel electrophoresis of PCR products for MMP-2 and MMP-9 in NSCLC cell-lines.

MMP-2 60°C 35 cycles (350bp)
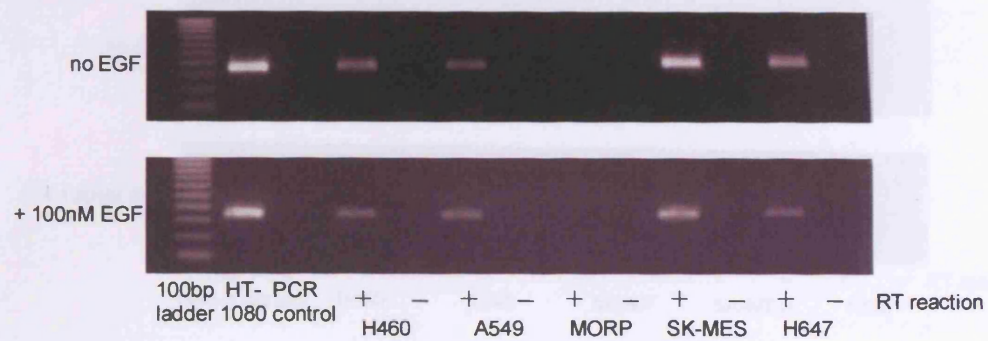
MMP-9 66°C 35 cycles (250bp)
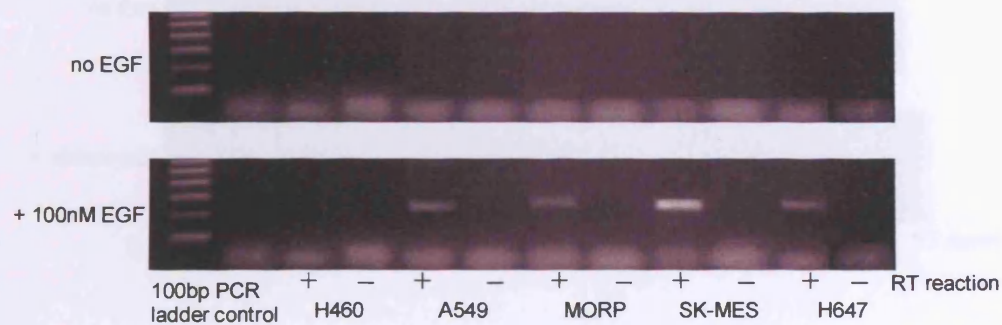
Figure 6.2 Immunohistochemistry for EGFR in H460 and A549 cell-lines.

a) H460 immunointensity score +
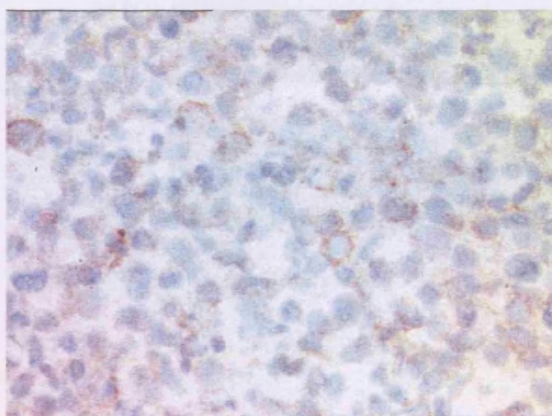
b) A549 immunointensity score +++

115
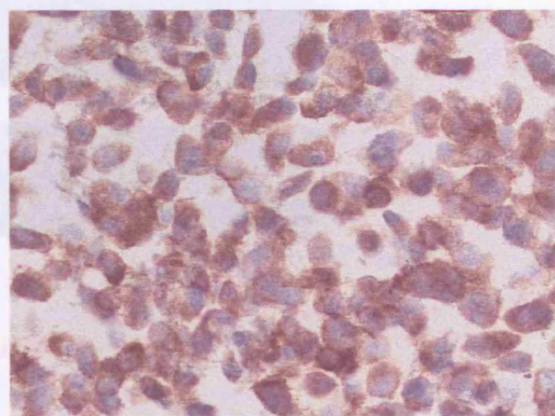
Figure 6.3 Agarose gel electrophoresis of PCR products for MT1-MMP, TIMP-1 and TIMP-2 in NSCLC cell-lines.

MT1-MMP 60°C 35 cycles (315bp)
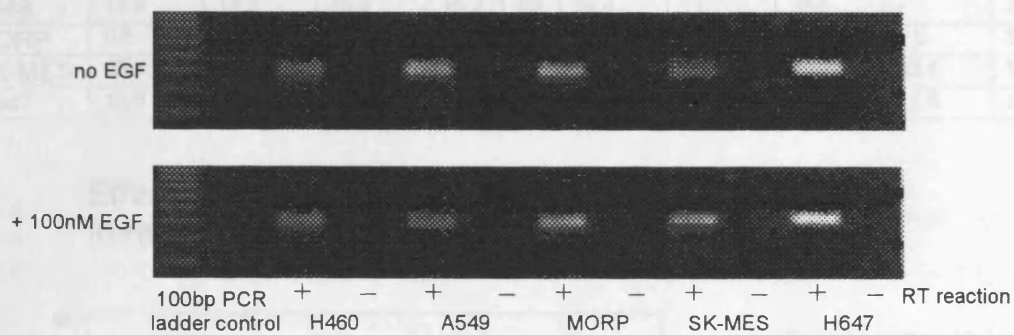
TIMP-1 59°C 30 cycles (350bp)
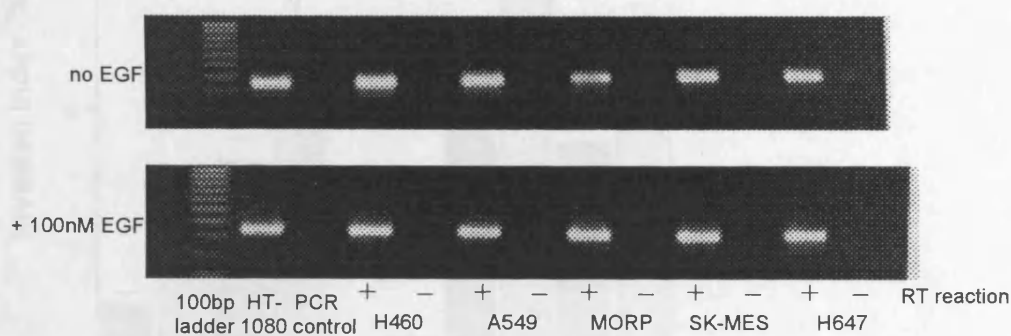
TIMP-2 59°C 30 cycles (250bp)
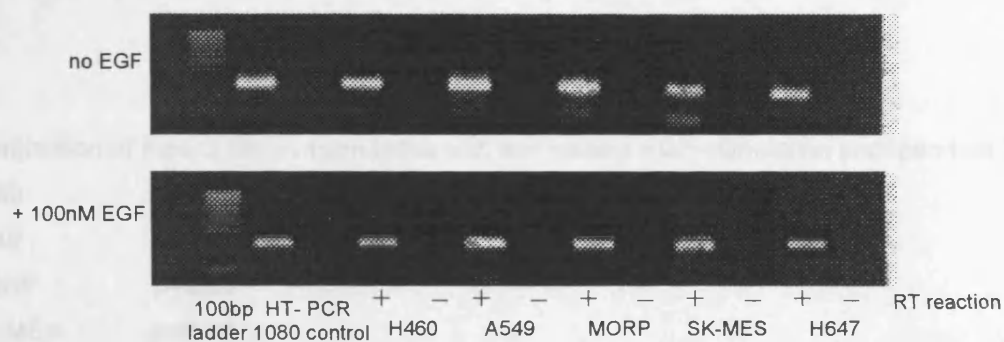
Figure 6.4 Effects of EGF on the invasion index in NSCLC cell-lines.

cell-line	Baseline invasion index				EGF-stimulated invasion index				
	Invasion Index 1	invasion index 2	invasion index 3	mean (SEM)	invasion index 1	invasion index 2	invasion index 3	Invasion index 4	mean (SEM)
H460	7.8	5.3	5.2	6.1 (0.85)	18.5	11.3	10.8	15.2	14.0 (1.81)
A549	18.9	13.2	16.6	16.2 (1.66)	38.4	11.7	27.5	-	25.9 (7.75)
MORP	0.5	1.8	1.6	1.3 (0.40)	0.0	5.5	7.2	1.5	3.6 (1.68)
SK-MES	23.4	27.6	15.7	22.2 (3.48)	21.7	18.8	39.3	43.6	30.8 (6.21)
H647	12.5	30.6	9.7	17.6 (6.55)	14.2	25.6	24.7	17.8	20.6 (2.75)

Effect of 48 hours 100nM EGF on the invasion index in NSCLC cell-lines

Error Bars show Mean +/- 1.0 SE

Bars show Means
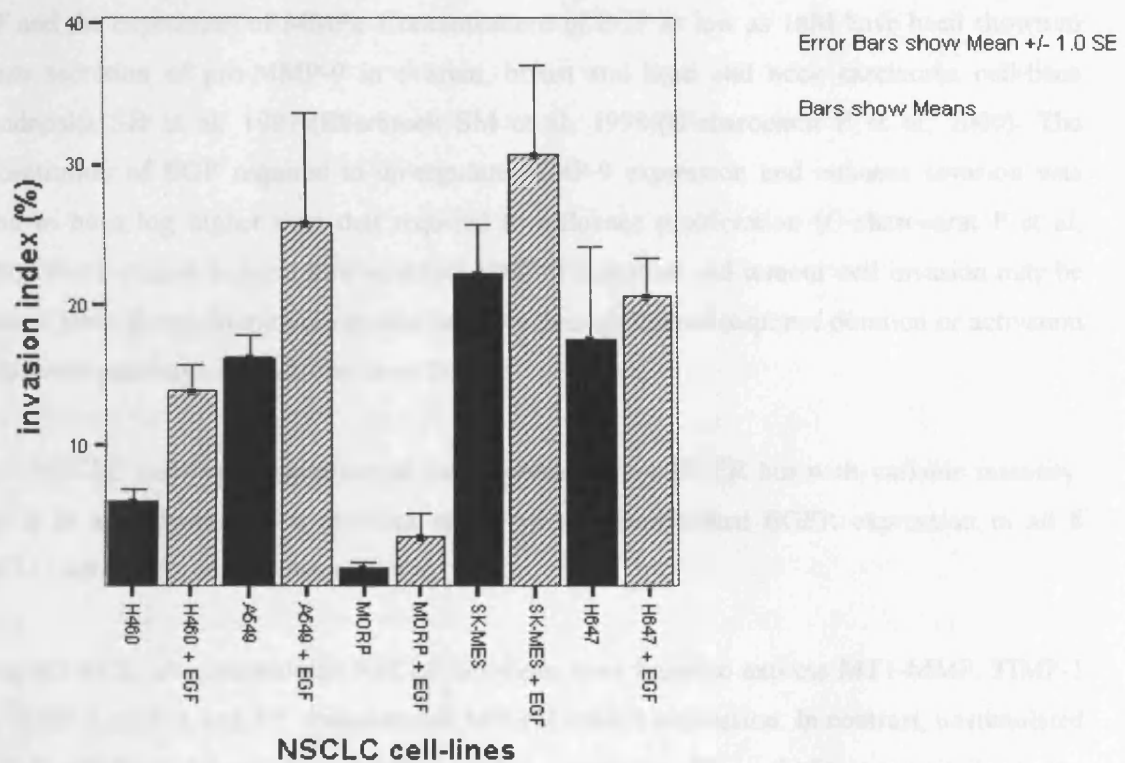
Comparison of means for invasion index with and without EGF-stimulation (independent T-test)

H460	p=0.016*
A549	p=0.34
MORP	p=0.28
SK-MES	p=0.29
H647	p=0.7

Discussion.

The processes of tumour invasion and angiogenesis, critical for tumour growth and metastasis, are facilitated by MMP-induced breakdown of the basement membrane and the ECM. MMPs may also release activated growth factors previously sequestrated in the ECM (Pepper MS et al, 1990) and so stimulate autocrine and paracrine growth factor signalling pathways, including EGFR activation.

Receptor-mediated induction of MMPs represents one possible mechanism linking receptors to tumour growth and metastasis. Recent studies have evaluated the possible correlation between EGF and the expression of MMPs. Concentrations of EGF as low as 1nM have been shown to induce secretion of pro-MMP-9 in ovarian, breast and head and neck carcinoma cell-lines (Kondapaka SB et al, 1997)(Ellerbroek SM et al, 1998)(O-charoenrat P et al, 2000). The concentration of EGF required to up-regulate MMP-9 expression and enhance invasion was found to be a log higher than that required to influence proliferation (O-charoenrat P et al, 2000). These studies suggest EGF-induced MMP-9 induction and tumour cell invasion may be separate from the proliferative response possibly through altered response duration or activation of different pathways downstream from EGFR.

All 5 NSCLC cell-lines demonstrated immunostaining for EGFR but with variable intensity. This is in agreement with a previous study which demonstrated EGFR expression in all 8 NSCLC cell-lines tested (Haeder M et al, 1988).

Using RT-PCR, all unstimulated NSCLC cell-lines were found to express MT1-MMP, TIMP-1 and TIMP-2 mRNA and 4/5 demonstrated MMP-2 mRNA expression. In contrast, unstimulated NSCLC cell-lines did not show MMP-9 mRNA expression. These findings are similar to the only other study of MMP and TIMP mRNA expression in NSCLC cell-lines (Suzuki M, 1999). They demonstrated MMP-2 mRNA in 5/5, MMP-9 mRNA in 1/5, TIMP-1 mRNA in 4/5 and TIMP-2 in 5/5 cell-lines.

Up-regulation of MMP-9 mRNA on exposure to 100nM EGF for 48 hours was demonstrated in 4 out of 5 NSCLC cell-lines without altering MMP-2 expression. Subjectively SK-MES and A549 cell-lines demonstrated strong +++ immunostaining for EGFR and appeared to have more intense MMP-9 bands on RT-PCR after EGF stimulation. In contrast the H460 cell-line had weak EGFR immunostaining and did not demonstrate MMP-9 mRNA expression after EGF stimulation. This finding suggests the number of EGFR receptors expressed on the cell surface may relate to the ability of EGF to up-regulate MMP-9 expression.

118

These results are in keeping with those in breast, ovarian and head and neck carcinomas and indicate that the MMP-9 gene is inducible, unlike that of MMP-2 (Benbow U et al, 1997). Activation of the EGFR appears to lead to up-regulation of MMP-9. The signal transduction mechanism may involve the MAP kinase pathway (Tanaka H et al, 1999)(Reddy KB et al, 1999)(Westermarck J et al, 1999) (figure 1.4) or the E-cadherin/β-catenin pathway as previously discussed (chapter 5 discussion) (Jones JL et al, 1996)(Llorens A et al, 1998).

The invasion index was higher in the four NSCLC cell-lines that expressed MMP-2 mRNA compared to the cell-line where MMP-2 mRNA was not demonstrated. This is in keeping with a previous enzyme-linked immunoassay study in NSCLC cell-lines in which the cell-lines with the highest invasive potential secreted the highest levels of MMP-2 (Zucker S et al, 1992). This suggests that MMP-2 may be more important than MMP-9 in invasion. EGFR may lead to increased activation of MMP-2 without upregulation of mRNA expression, possibly via the induction of COX-2 (Tsuji M et al, 1997)(Takahashi Y et al, 1999). In this study the invasion index was higher in all cell-lines after EGF stimulation although only significantly so for H460. Coincidentally this was the cell-line that did not demonstrate EGF-induced MMP-9 mRNA. There were problems with the membrane becoming loose from the well-insert and potentially altering the gradient between the conditioned media and the inside of the well. This could allow for some of the apparently aberrant results in similar condition experiments and therefore significantly alter the invasion index. Those cell-lines with the lowest invasion index (MOR-P and H460) would potentially be most affected by this. The technique required to coat the PET-membrane with an even and complete layer of Matrigel will improve with experience. Further assays will need to be performed using a new batch of inserts to fully evaluate the effects of EGF on invasion.

Our previous immunohistochemical studies showed a significant relationship between both membranous and overall EGFR expression and tumour cell MMP-9 expression in NSCLC tumours. This study demonstrates EGFR-stimulation by EGF leads to the up-regulation of MMP-9 mRNA in NSCLC cell-lines. The number of receptors present on the surface of the cell may dictate this response. Together these findings suggest that EGFR is involved in the specific up-regulation of MMP-9. These results add further support the potential of MMP inhibitors (Macaulay VM et al, 1999), EGFR antibodies (Bunn PA et al, 2000) and EGFR tyrosine kinase inhibitors (Lei W et al, 1999) in the treatment of NSCLC.

Chapter 7

Final Discussion and Future Directions

Final Discussion and Future Directions

This thesis has examined, using immunohistochemistry, the expression, interrelationships and correlation with survival of four potential proliferative, invasive and metastatic mechanisms in a series of NSCLC tumours in order to construct a molecular staging model. The impact of EGF on both the expression of the gelatinase family mRNA and the invasiveness in five NSCLC cell-lines was studied.

Angiogenesis, assessed indirectly using Chalkley counting of microvessels, was found to be an independent prognostic marker in agreement with many previous studies of solid tumours including NSCLC. A recent study suggests an existing vascular bed may be exploited by a NSCLC tumour without the need for new vessel formation, and that these tumours have a worse prognosis (Pezzella F et al, 1997). CD34 is a pan endothelial marker and does not differentiate if a vessel in new or old. The integrin $\alpha_v\beta_3$ is up-regulated in activated endothelial cells, a requirement of angiogenesis, and is therefore a potential marker of new vessel formation (Brooks PC et al, 1994). We are currently investigating the relationship between CD34 endothelial vessel counts and $\alpha_v\beta_3$ vessel counts in normal lung and NSCLC tumour specimens and assessing their impact on survival in a prospective series of resected NSCLC tumours. The evaluation of the angiogenic growth factors VEGF, PD-ECGF and bFGF and their association with the microvessel count are still to be evaluated in this series.

Bcl-2 expression was found to be associated with a good prognosis in NSCLC. The expression of Bcl-2 in dysplastic bronchial cells increases as the dysplasia progresses and then may be lost in overtly malignant cells. These findings suggest that tumours retaining Bcl-2 expression, and conferring a better prognosis, may have less molecular alterations than tumours that have lost Bcl-2 expression. A sub-group of tumours that demonstrated strong immunoreactivity for Bcl-2 had a worse outcome than the weakly staining cases. The strongly staining cases may represent amplification or up-regulation of Bcl-2, possibly in response to other oncogenes. Although no association was seen between p53 and Bcl-2 expression, further studies into other apoptotic proteins such as Bax, Bak and MDM-2 could be performed. Potentially the expression of these proteins could be correlated with the apoptotic index of the tumour.

Both membranous and cytoplasmic EGFR expression was seen in NSCLC. Although no pattern of EGFR expression was found to correlate with prognosis, overall and membranous EGFR expression were inversely related to the microvessel count. This finding is not in agreement with *in vitro* studies that suggest EGFR is pro-angiogenic. However this is not the first study in NSCLC to suggest an inverse association between erb receptors and angiogenesis

(Giatromanolaki A et al, 1996b). This raises the possibility that high levels of erb receptor expression may allow a tumour cell to survive and invade in the absence of angiogenesis. This also highlights a possible problem with looking at pathways *in vitro* and assuming similar results will occur *in vivo*.

The expression of c-erbB-2 was evaluated using the semi-quantitative HercepTest™. Only a small percentage of NSCLC tumours demonstrated membranous staining, the percentage increasing in higher pathological stages. The reproducibility of HercepTest™ staining was confirmed with identical scoring of 2+ and 3+ cases performed in a separate laboratory. FISH analysis showed that HercepTest™ 3+ staining was specific for gene amplification. This study suggests that, in contrast to breast cancer, only a small percentage of operable NSCLC patients may benefit from adjuvant Herceptin™ treatment. A higher percentage of stage IV NSCLC tumours demonstrated HercepTest™ positivity and there may be a role for Herceptin™ in the treatment of unresectable disease. An evaluation of the efficacy of Herceptin™ in this group of patients, especially in combination with palliative chemotherapy, should be carried out.

MMP-2, MMP-9 and TIMP-2 were widely expressed in both the tumour cells and the surrounding stroma of NSCLC tumours. Tumour cell MMP-9 expression was shown to be an independent marker of poor prognosis and was significantly correlated with EGFR immunoreactivity. Tumours expressing both tumour cell MMP-9 and EGFR had a particularly poor outcome. TIMP-2 tumour cell expression was inversely related with EGFR. This may be due to EGFR up-regulating MMP-9 whilst down-regulating TIMP-2 expression. The inverse relationship between membranous EGFR expression and the microvessel count, the positive correlation between EGFR and MMP-9 and the independent prognostic significance of both microvessel count and EGFR/MMP-9 suggests that angiogenesis and EGFR/MMP-9 may be two separate pathways in tumour invasion and metastasis. Further immunohistochemical work will evaluate MT1-MMP and TIMP-1 expression in this same series of tumours.

There was no association between MMP-2, MMP-9 or TIMP-2 immunoreactivity or any combination of these three factors and the microvessel count. Although MMPs have been shown to have an important role in the angiogenic process (Johnson MD et al, 1994) there is considerable overlap in their substrate specificity and it may be that various combinations of MMPs are capable of supporting angiogenesis.

A stage-independent prognostic model using the four immunohistochemical markers CD34, Bcl-2, EGFR and MMP-9 performed on routinely processed tissue was developed. This model identified patients at high risk of recurrence who may benefit from adjuvant treatment with

traditional cytotoxic chemotherapy. These patients could be treated on an individual basis with the novel targeted therapeutic agents such as MMP (Macaulay VM et al, 1999) or EGFR tyrosine kinase (Bunn PA et al, 2000) inhibitors. A prospective study of this model, preferably multicentre, needs to be carried out. Evaluation of the efficacy of the novel agents in NSCLC, especially those tumours expressing MMPs and EGFR is merited. Further refinement of this model with the addition of other factors may add to the prognostic value but will also detract from its simplicity.

Established NSCLC cell-lines were used to examine more directly the relationship between invasion and gelatinase family mRNA expression. *In vitro* studies demonstrated 4/5 cell-lines expressed MMP-2 mRNA and all five cell-lines expressed MT1-MMP, TIMP-1 and TIMP-2 mRNA. Stimulation with 100nM EGF for 48 hours up-regulated MMP-9 mRNA expression in 4/5 NSCLC cell-lines where previously MMP-9 mRNA had not been demonstrated. EGF had no obvious effect on MMP-2, MT1-MMP, TIMP-1 or TIMP-2 mRNA expression. Immunohistochemistry revealed EGFR was expressed by all 5 cell-lines. There appeared to be a relationship between EGFR immunointensity and the intensity of the EGF-induced MMP-9 band on RT-PCR. Zymography of the cell media for MMP-2 and MMP-9 will show if MMP-9 mRNA up-regulation actually leads to MMP-9 protein secretion. In association with the *in vivo* finding of a significant correlation between MMP-9 and EGFR expression, the *in vitro* results suggest EGFR is involved in the up-regulation of MMP-9 and further supports the use of MMP inhibitors and EGFR tyrosine kinase antagonists in the treatment of NSCLC.

Although the invasion index was increased in all cell-lines after stimulation with EGF this increase reached significance in one cell-line only. The variability in results between assays may in part be due to problems with the membranes becoming detached from the well-inserts and therefore these experiments would merit repeating with a new batch of inserts.

Future studies will see if EGFR tyrosine antagonists and EGFR antibodies inhibit MMP-9 up-regulation. Although previous studies have evaluated the MAP kinase signalling pathway between EGFR and MMP-9, there is evidence from studies in breast and skin cancer to suggest that the E-cadherin/β-catenin system may be involved (Jones JL et al, 1996)(Llorens A et al, 1998). This is further supported by the recent study showing stage I NSCLC tumours with a high MMP-2 and MMP-9:E-cadherin mRNA ratio were at increased risk of relapse after surgery (Herbst RS et al, 2000). Immunohistochemical studies on NSCLC cell-lines will see if EGF leads to down-regulation of E-cadherin, the nuclear appearance of β-catenin and altered morphology of cells. Further studies could see if the addition of E-cadherin antibodies to cell-

lines alters MMP-9 expression. Furthermore COX-2 is involved with tumour growth, invasion and angiogenesis (Tsuji M et al, 1998). Assessment of COX-2 expression in the NSCLC cell-lines before and after EGF stimulation and COX-2 inhibition will assess the impact of this immunomodulatory factor upon invasion, MMP-9 regulation and E-cadherin expression.

Scientific research continues to offer potential targets for clinical manipulation in the treatment of cancer. Hopefully it will not be long before increasing knowledge of the mechanisms of tumour invasion and metastasis in NSCLC passes from the bench to the bedside and that major advances in patient survival begin to appear.

References

References

Ahonen M, Baker AH, Kahari V-M (1998). Adenovirus-mediated gene delivery of tissue-inhibitor of metalloproteinases-3 inhibits invasion and induces apoptosis in melanoma cells. Cancer Res 58:2310-2315

Aikawa H, Takahashi H, Fujimura S, et al (1999). Immunohistochemical study on tumour angiogenic factors in non-small cell lung cancer. Anticancer Res 19:4305-4309

Albini A, Iwamoto Y, Kleinman HK, et al (1987). A rapid *in vitro* assay for quantitating the invasive potential of tumor cells. Cancer Res 47:3239-3245

Alder I (1912). Primary malignant growths of the lungs and bronchi: a pathological and clinical study. Longmans, Green and Co., London

Allam HS, Arand G, Lippman ME, et al (1993). Association of MMP-2 activation potential with metastatic progression in human breast cancer cell lines independent of MMP-2 production. J Natl Cancer Inst 85:1758-1764

Allred DC, Swanson PE (2000). Testing for erbB-2 by immunohistochemistry in breast cancer. Am J Clin Pathol 113:171-5

Amstad PA, Lik H, Ichimiya M, et al (1997). bcl-2 enhancement of malignant transformation in mouse epidermal JB-6 cells. Mol Carcinogenesis 20:231-239

Amour A, Slocombe PM, Webster A, et al (1998). TNF-α converting enzyme (TACE) is inhibited by TIMP-3. FEBS Lett 435:39-44

An WG, Kanakal M, Celeste Simon M, et al (1998). Stabilization of wild-type p53 by hypoxia-inducible factor-1α. Nature 392:405-408

An Z, Wang X, Willmott N, et al (1997). An orally-active synthetic MMP inhibitor CT1746 reduces tumour spread and metastasis converting aggressive colonic cancer into a more indolent disease in a nude-mice model. Clin Exp Metastasis 15:184-195

Anand-Apte B, Pepper MS, Voest E, et al (1997). Inhibition of angiogenesis by tissue inhibitor of metalloproteinase-3. Investig Ophthalmol Vis Sci 38:817-823

Anderson IC, Shipp MA, Docherty AJP, et al (1996). Combination therapy including a gelatinase inhibitor and cytotoxic agent reduces local invasion and metastases of murine Lewis lung carcinoma. Cancer Res 56:715-720

Angeletti CA, Lucchi M, Fontanini G, et al (1996). Prognostic significance of tumoral angiogenesis in completely resected late stage lung carcinoma (stage IIIA-N2). Cancer 78:409-415

Anton RC, Brown RW, Younes M, et al (1997). Absense of prognostic significance of bcl-2 immunopositivity in non-small cell lung cancer: Analysis of 427 cases. Hum Pathol 28:1079-1082

Apolinario RM, van der Valk P, de Jong JS, et al (1997). Prognostic value of the expression of p53, *bcl-2*, and *bax* oncoproteins, and neovascularization in patients with radically resected non-small-cell lung cancer. J Clin Oncol 15:2456-2466

Apte SS, Olsen BR, Murphy G (1995). The gene structure of tissue inhibitot of metalloproteinase (TIMP) -3 and its inhibitory activities define the distinct timp gene family. J Biol Chem 270:14313-14318

Arenberg DA, Kunkel SL, Polverini PJ, et al (1996). Inhibition of interleukin-8 reduces tumorigenesis of human non-small cell lung cancer in SCID mice. J Clin Invest 97:2792-2802

Arenberg DA, Polverini PJ, Kunkel SL, et al (1997). The role of CXC chemokines in the regulation of angiogenesis in non-small cell lung cancer. J Leukocyte Biol 62:554-562

Arenberg DA, Keane MP, DiGiovine B, et al (1998). Epithelial-neutrophil activating peptide (ENA-78) is an important angiogenic factor in non-small cell lung cancer. J Clin Invest 102:465-472

Ashara T, Bauters C, Zheng LP, et al (1995). Synergistic effect of vascular endothelial growth factor and basic fibroblast growth factor on angiogenesis in vivo. Circulation 92:II-365-II-371

Atula S, Grenman R, Syrjanen S (1997). Fibroblasts can modulate the phenotype of malignant epithelial cells in vitro. Exp Cell Res 235:180-187

Autio Harmainen H, Kartunnen T, Hurskainen T, et al (1993). Expression of 72 kilodalton type IV collagenase (gelatinase A) in benign and malignant ovarian tumours. Lab Invest 69:312-321

Ayhan A, Yasui W, Yokozaki H, et al (1994). Loss of heterozygosity at the bcl-2 gene locus and expression of bcl-2 in human gastric and colorectal carcinomas. Jpn J Cancer 85:584-591

Bafetti LM, Young TN, Itoh Y, et al (1998). Intact vitronectin induces matrix metalloproteinase-2 and tissue inhibitor of metalloproteinases-2 expression and enhanced cellular invasion by melanoma cells. J Biol Chem 273:143-149

Baker AH, Zaltsman AB, George SJ, et al (1998). Divergent effects of tissue inhibitors of metalloproteinase-1, -2 or –3 overexpression on rat vascular smooth muscle cell invasion, proliferation and death in vitro: TIMP-3 promotes apoptosis. J Clin Invest 101:1478-1487

Ballin M, Gomez DE, Sinha CC, et al (1988). *Ras* oncogene mediated induction of a 92 kDa metalloproteinase: strong correlation with a malignant phenotype. Biochem Biophys Res Commun 154:832-838

Barsky SH, Siegal GP, Jannotta F, et al (1993). Loss of basement membrane components by invasive tumours but not by their benign counterparts. Lab Invest 49:140-147

Baselga J, Norton L, Albanell J, et al (1998). Recombinant humanized anti-HER2 antibody (HERCEPTIN®) enhances the activity of paclitaxel and doxorubicin against HER2/neu overexpressing human breast cancer xenografts. Cancer Res 58:2825-31

Basset P, Bellocq JP, Wolf C, et al (1990). A novel metalloproteinase gene specifically expressed in stromal cells of breast carcinomas. Nature 348:699-704

Bauer J, Margolis M, Schreiner C, et al (1992). *In vitro* model of angiogenesis using a human endothelial-derived permanent cell line: contributions of induced gene expression, G-proteins and integrins. J Cell Physiol 153:437-449

Becker D, Meier CB, Herlyn M (1989). Proliferation of human malignant melanomas is inhibited by antisense oligodeoxynucleotides against basic fibroblast growth factor. EMBO J 8:3685-3691

Benefield J, Petruzzelli GJ, Fowler S, et al (1996). Regulation of the steps of angiogenesis by human head and neck squamous cell carcinomas. Invasion Metastasis 16:291-301

Benbow U, Brinckerhoff C (1997). The AP-1 site and MMP gene regulation: What is all the fuss about? Matrix Biol 519-526

Bennett WP, Colby TV, Travis WT, et al (1993). p53 accumulates frequently in early bronchial neoplasia. Cancer Res 53:4817-4822

Bernhard EJ, Gruber SB, Muschel RJ (1994). Direct evidence linking expression of metalloproteinase 9 (92-kDa gelatinase/collagenase) to the metastatic phenotype in transformed rat embryo cells. Proc Nat Acad Sci 91:4293-4297

Bertaux B, Hornebeck W, Eisen AZ, et al (1991). Growth stimulation of human keratinocytes by tissue inhibitor of metalloproteinases. J Invest Dermatol 97:679-685

Bhargava V, Kell DL, van de Rijn M, et al (1994). Bcl-2 immunoreactivity in breast carcinomas correlates with hormone receptor positivity. Am J Pathol 145:535-540

Bian J, Wang Y, Smith MR, et al (1996). Supression of in vivo tumour growth and induction of suspension cell death by tissue inhibitor of metalloproteinases (TIMP) –3. Carcinogenesis 17:1805-1811

Bian J, Sun Y (1997). Transcriptional activation by p53 of the human type IV collagenase (gelatinase A or matrix metalloproteinase 2) promoter. Mol Cell Biol 17:6330-6338

Bigg HF, Shi YE, Liu YE, et al (1997). Specific, high affinity binding of tissue inhibitor of metalloproteinases-4 (TIMP-4) to the COOH-terminal hemopexin-like domain of human gelatinase A. TIMP-4 binds progelatinase A and the COOH-terminal domain in a similar manner to TIMP-2. J Biol Chem 272:15496-15500

Birkedal-Hansen H, Moore WG, Bodden MK, et al (1993). Matrix metalloproteinases: a review. Crit Rev Oral Biol Med 4:197-250

Blancher C, Harris AL (1998). The molecular basis of the hypoxia response pathway: tumour hypoxia as a therapeutic target. Cancer and Metastasis Rev 17:187-194

Blezinger P, Wang J, Gondo M, et al (1999). Systemic inhibition of tumour growth and tumour metastases by intramuscular administration of the endostatin gene. Nature Biotech 17:343-348

Boag AH, Young ID (1994). Increased expression of the 72-kd type IV collagenase in prostatic adenocarcinoma. Demonstration by immunohistochemistry and in-situ hybridization. Am J Pathol 144:585-591

Boehm T, Folkman J, Browder T, et al (1997). Antiangiogenic therapy of experimental cancer does not induce acquired drug resistance. Nature 390:404-407

Bolon I, Brambilla E, Vandenbunder B, et al (1996). Changes in the expression of matrix proteases and of the transcription factor c-Ets-1 during progression of precancerous bronchial lesions. Lab Invest 75:1-13

Bolon I, Devouassoux M, Robert C, et al (1997). Expression of urokinase-type plasminogen activator, stromelysin-1, stromelysin-3 and matrilysin genes in lung carcinomas. Am J Pathol 150:1619-1629

Bouck N, Stellmach V, Hsu SC (1996). How tumours become angiogenic. Adv Cancer Res 69:135-174

Boudreau N, Sympson CJ, Werb Z, et al (1995). Suppression of ICE and apoptosis in mammary epithelial cells by extracellular matrix. Science 267:891-895

Boyle P (1997). Cancer, cigarette smoking and premature death in Europe: a review including the recommendations of European Cancer Experts Consensus Meeting, Helsinki, October 1996. Lung Cancer 17:1-60

Brock TA, Dvorak HF, Senger DR (1991). Tumour secreted vascular permeability factor increases cytosolic Ca2+ and von Willebrand factor release in human endothelial cells. Am J Pathol 138:213-221

Brooks PC, Clark RAF, Cheresh DA (1994). Requirement of vascular integrin alpha-v/beta-3 for angiogenesis. Science 264:569-571

Brooks PC, Stromblad S, Sanders LC, et al (1996). Localization of matrix metalloproteinase MMP-2 to the surface of invasive cells by interaction with integrin alpha-v/beta-3. Cell 85:683-693

Brown LF, Berse B, Jackman RW, et al (1993a). Expression of vascular permeability factor (vascular endothelial factor) and its receptors in adenocarcinomas of the gastrointestinal tract. Cancer Res 1993;53:4727-4735

Brown LF, Berse B, Jackman RW, et al (1993b). Increased expression of vascular permeability factor (vascular endothelial factor) and its receptors in kidney and bladder carcinomas. Am J Pathol 143:1255-1262

Brown LF, Berse B, Jackman RW, et al (1995). Expression of vascular permeability factor (vascular endothelial growth factor) and its receptors in breast cancer. Hum Pathol 26:86-91

Brown PD, Bloxidge RE, Stuart NSA, et al (1993). Association between expression of 72-kilodalton gelatinase and tumour spread in non-small cell lung cancer. J Nat Cancer Inst 85:574-578

Bruns CJ, Solorzano CC, Harbison MT, et al (2000). Blockade of the epidermal growth factor receptor signaling by a novel tyrosine kinase inhibitor leads to apoptosis of endothelial cells and therapy of human pancreatic carcinoma. Cancer Res 60:2926-2935

Buadu L, Murakami J, Murayama S, et al (1996). Breast lesions: correlation of contrast medium enhancement patterns on MR images with histopathological findings and tumor angiogenesis. Radiology 1996;200:639-649

Bunn PA, Soriano A, Johnson G, et al, (2000). New therapeutic strategies for lung cancer. Chest 117;163S-168S.

Canman CE, Gilmer TM, Coutts SB, et al (1995). Growth factor modulation of p53-mediated growth arrest versus apoptosis. Genes Dev 9:600-611

Cannette-Soler R, Litzky L, Lubensky I, et al (1994). Localization of the 92-kD gelatinase mRNA in squamous cell and adenocarcinomas of the lung using in-situ hybridization. Am J Pathol 144:518-527

Carmeliet P, Dor Y, Herbert J-M, et al (1998). Role of HIF-1α in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis. Nature 394:485-490

Carrere S, Verger A, Fluorens A, et al (1998). Erg proteins, transcription factors of the Ets family, form homo, heterodimers and ternary complexes via two distinct domains. Oncogene 16:3261-3268

Caudroy S, Polette M, Tournier JM, et al (1999). Expression of the extracellular matrix metalloproteinase inducer (EMMPRIN) and the matrix metalloproteinase-2 in bronchopulmonary and breast lesions. J Histochem Cytochem 47:1575-1580

Chambers AF, Matrisian LM (1997). Changing views of the role of matrix metalloproteinases in metastasis. J Nat Canc Inst 89:1260-1270

Chandrachud LM, Pendleton N, Chisholm DM, et al (1997). Relationship between vascularity, age and survival in non-small cell lung cancer. Br J Cancer 76:1367-1375

Charnock-Jones DS, Sharkey AM, Boocock CA, et al (1994). Vascular endothelial growth factor receptor localisation and activation in human trophoblast and choriocarcinoma cells. Biol Reprod 51:524-530

Cheng SY, Huang HJ, Nagane, et al (1996). Suppression of glioblastoma angiogenicity and tumorigenicity by inhibition of endogenous expression of vascular endothelial growth factor. Proc Natl Acad Sci USA 93:8502-8507

Chinje EC, Patterson AV, Saunders MP, et al (1999). Does reductive metabolism predict response to tirapazamine (SR 4233) in human non-small-cell lung cancer cell lines? Br J Cancer 81:1127-1133

Chiou S-K, Rao L, White E (1994). Bcl-2 blocks p53-dependent apoptosis. Mol Cell Biol 14:2556-2563

Chirivi RG, Garofalo A, Crimmin MJ, et al (1994). Inhibition of the metastatic spread and growth of B16-BL6 murine melanoma by a synthetic matrix metalloproteinase inhibitor. Int J Cancer 58:460-464

Ciardiello F, Bianco R, Damiano V, et al (1999). Antitumor activity of sequential treatment with topotecan and anti-epidermal growth factor receptor monoclonal antibody C225. Clin Cancer Res 5:909-16

Clark IM, Powell LK, Cawston TE (1994). Tissue inhibitor of metalloproteinases (TIMP-1) stimulates secretion of collagenase from human skin fibroblasts. Biochem Biophys Res Comm 203:874-880

Claus M, Weich H, Breier G, et al (1996). The vascular endothelial growth factor receptor Flt-1 mediates biological activity. Implications for the functional role of placental growth factor in monocyte activation and chemotaxis. J Biol Chem 271:17629-17634

Cobleigh MA, Vogel CL, Tripathy D, et al (1999). Multinational study of the efficacy and safety of humanized anti-HER2 monoclonal antibody in women who have HER2-overexpressing metastatic breast cancer that has progressed after chemotherapy for metastatic disease. J Clin Oncol 17:2639-48

Collier IE, Wilhelm SM, Eisen AZ, et al (1988). H-ras oncogene-transformed bronchial epithelial cells (TBE-1) secrete a single metalloproteinase capable of degrading basement collagen. J Biol Chem 263:6579-6587

Conn G, Bayne M, Soderman L, et al (1990). Amino acid and cDNA sequence of a vascular endothelial cell mitogen homologous to platelet-derived growth factor. Proc Natl Acad Sci USA 87:2628-2632

Cozzolino F, Torcia M, Lucibello M, et al (1993). Cytokine-mediated control of endothelial cell growth: interferon-α and interleukin-2 synergistically enhance basic fibroblast growth factor synthesis and induce release promoting cell growth in vitro and in vivo. J Clin Invest 91:2054-2512

Crickard K, Gross JL, Crickard U, et al (1994). Basic fibroblast growth factor and receptor expression in human ovarian cancer. Gynecol Oncol 55:277-284

Crowe P, Walter BN, Mohler KM, et al (1995). A metalloproteinase inhibitor blocks shedding of the 80-kD TNF receptor and TNF processing in T lymphocytes. J Exp Med 181:1205-1210

Czubayko F, Smith RV, Chung HC, et al (1994). Tumour growth and angiogenesis induced by a secreted binding protein for fibroblast growth factors. J Biol Chem 269:28243-28248

Czubayko F, Liaudet-Coopman EDE, Aigner A, et al (1997). A secreted FGF-binding protein can serve as the angiogenic switch in human cancer. Nature Med 3:1137-1140

Dalquen P, Sauter G, Torhorst J, et al (1996). Nuclear p53 overexpression is an independent prognostic parameter in node-negative non-small cell lung carcinoma. J Pathol 178:53-58

Dameron KM, Volpert OG, Tainsky MA, et al (1994). Control of angiogenesis in fibroblasts by p53 regulation of thrombospondin-1. Science 265:1582-1584

Davies B, Miles DW, Happerfield MLC, et al (1993a). Activity of type IV collagenases in benign and malignant breast disease. Br J Cancer 67:1126-1131

Davies B, Waxman J, Harpret W, et al (1993b). Levels of matrix metalloproteinases in bladder cancer correlate with tumour grade and invasion. Cancer Res 53:1-5

Dazzi C, Cariello A, Maioli P, et al (1999). Prognostic and predictive value of intratumoral microvessels density in operable non-small-cell lung cancer. Lung Cancer 24:81-88

Decaussin M, Sartelet H, Robert C, et al (1999). Expression of vascular endothelial growth factor (VEGF) and its two receptors (VEGF-R1-Flt1 and VEGF-R2-Flk1/KDR) in non-small cell carcinomas (NSCLCs): correlation with angiogenesis and survival. J Pathol 188:369-377

De Clerck YA (1988). Purification and characterization of a collagenase inhibitor produced by bovine vascular smooth muscle cells. Arch Biochem Biophys 265:28-37

De Clerk YA, Perez N, Schimar H, et al (1992). Inhibition of invasion and metastasis in cells transfected with an inhibitor of metalloproteinases. Cancer Res 52:701-708

Delebecq TJ, Porte H, Zerimech F, et al (2000). Overexpression level of stromelysin 3 is related to the lymph node involvement in non-small cell lung cancer. Clin Cancer Res 6:1086-1092

deVries C, Escobedo JA, Ueno H, et al (1992). The fms-like tyrosine kinase, a receptor for vascular endothelial growth factor. Science 255:989-991

Diez M, Pollan M, Maestro M, et al (1997). Prediction of recurrence by quantification of p185^{neu} protein in non-small-cell lung cancer tissue. Br J Cancer 75:684-689

Dobbs SP, Hewett PW, Johnson IR, et al (1997). Angiogenesis is asociated with vascular endothelial growth factor expression in cervical intraepithelial neoplasia. Br J Cancer 76:1410-1415

Docherty AJP, Lyons A, Smith BJ, et al (1985). Sequence of human tissue inhibitor of metalloproteinases and its identity to erythroid-potentiating activity. Nature 318:66-69

Dong Z, KumarR, Fidler IJ (1996). Generation of the angiogenesis inhibitor, angiostatin, by Lewis lung carcinoma is mediated by macrophage elastase. Proc Am Assoc Cancer Res 37:403a

Duarte IG, Bufkin BL, Pennington MF, et al (1998). Angiogenesis as a predictor of survival after surgical resection for stage I non-small cell lung cancer. J Cardio Thorac Surg 115:652-659

Duffy CP, Elliott CJ, O'Connor RA, et al (1998). Enhancement of chemotherapeutic drug toxicity to human tumour cells in vitro by a subset of non-steroidal anti-inflammatory drugs (NSAIDs). Eur J Cancer 34:1250-1259

Dvorak HF, Brown LF, Detmar M, et al (1995). Vascular permeability factor/vascular endothelial growth factor, microvascular permeability, and angiogenesis. Am J Pathol 36:139-155

Eccles SA, Box GM, Court WJ, et al (1996). Control of lymphatic and haematogenous metastasis of a rat mammary carcinoma by the matrix metalloproteinase inhibitor batimastat (BB-94). Cancer Res 54:4726-4728

Eccles SA (2000). Cell biology of lymphatic metastasis. The potential role of c-erbB oncogene signalling. Recent Results Cancer Res 157:41-54

Ellerbroek SM, Hudson LG, Stack MS (1998). Proteinase requirements of epidermal growth factor-induced ovarian cancer cell invasion. Int J Cancer 78:331-337

Evan GI, Wyllie AH, Gilbert CS, et al (1992). Induction of apoptosis in fibroblasts by c-myc protein. Cell 69:119-128

Favoni RE, de Cupis A, Ravera F, et al (1994). Expression and function of the insulin-like growth factor I system in human non-small-cell lung cancer and normal lung cell lines. Int J Cancer 56:858-866

Fisher C, Gilbertson-Beadling S, Powers EA, et al (1994). Interstitial collagenase is required for angiogenesis in vitro. Dev Biol 162:499-510

Fisseler-Eckhoff A, Rothstein D, Muller KM (1996). Neovascularization in hyperplastic, metaplastic and potentially preneoplastic lesions of the bronchial mucosa. Virchows Arch 429:95-100

Fleming MV, Guinee DG, Chu WS, et al (1998). Bcl-2 immunohistochemistry in a surgical series of non-small cell lung cancer patients. Human Pathol 29:60-64

Folkman J (1991). What is the evidence that tumors are angiogenesis-dependent? J Natl Cancer Inst 82:4-6

Fong G-H, Rossant J, Getenstein M, et al (1995). Role of Flt-1 receptor tyrosine kinase in regulation of assembly of vascular endothelium. Nature 376:66-67

Fong KM, Kida Y, Zimmerman PV, et al (1996). TIMP-1 and adverse prognosis in non-small cell lung cancer. Clin Cancer Res 2:1369-1372

Fong KM, Kida Y, Zimmerman PV, et al (1996). TIMP-1 and adverse prognosis in non-small cell lung cancer. Clin Cancer Res 2:1369-1372

Fontanini G, Vignati S, Bigini D, et al (1995a). Microvessel count predicts metastatic disease and overall survival in non-small-cell lung carcinomas. J Pathol 177:57-63

Fontanini G, Vignati S, Bigini D, et al (1995b). Bcl-2 protein: A prognostic factor inversely correlated to p53 in non-small cell lung cancer. Br J Cancer 71:1003-1007

Fontanini G, Vignati S, Bigini D, et al (1995c). Epidermal growth factor receptor (EGFr) expression in non-small cell lung carcinomas correlates with metastatic involvement of hilar and mediastinal lymph nodes in the squamous subtype. Eur J Cancer 31A:178-183

Fontanini G, Vignati S, Bigini D, et al (1996). Neoangiogenesis: a putative marker of malignancy in non-small-cell lung cancer (NSCLC) development. Int J Cancer 67:615-619

Fontanini G, Lucchi M, Vignati S, et al (1997a). Angiogenesis as a prospective indicator of survival in non-small-cell lung carcinoma: a prospective study. J Natl Cancer Inst 89:881-886

Fontanini G, Vignati S, Boldrini L, et al (1997b). Vascular endothelial cell growth factor is associated with neovascularization and influences progression of non-small cell lung carcinoma. Clin Cancer Res 1997;3:861-865

Fontanini G, Vignati S, Lucchi M, et al (1997c). Neoangiogenesis and p53 protein in lung cancer: their prognostic role and their relation with vascular endothelial growth factor (VEGF) expression. Br J Cancer 75:1295-1301

Fontanini G, De Laurentiis M, Vignati S, et al (1998a). Evaluation of epidermal growth factor-related growth factors and receptors and of neoangiogenesis in completely resected stage I-IIIa non-small-cell lung cancer: amphiregulin and microvessel count are independent prognostic indicators in survival. Clin Cancer Res 4:241-249

Fontanini G, Boldrini L, Vignati S, et al, (1998b). Bcl2 and p53 regulate vascular endothelial growth factor (VEGF)-mediated angiogenesis in non-small cell lung carcinoma. Eur J Cancer 34:718-723

Fontanini G, Boldrini L, Chine S, et al (1999). Expression of vascular endothelial growth factor mRNA in non-small cell lung carcinomas. Br J Cancer 79: 363 – 369

Fox SB, Smith K, Hollyer J, et al (1994). The epidermal growth factor receptor as a prognostic marker: results of 370 patients and review of 3009 patients. Breast Cancer Res and Treatment 29:41-49

Fox SB, Leek RD, Weekes MP, et al (1995). Quantification and prognostic value of breast cancer angiogenesis: comparison of microvessel density, Chalkley count and computer image analysis. J Pathol 177:275-283

Fox SB, Westwood M, Moghaddam A, et al (1996). The angiogenic factor platelet-derived endothelial cell growth factor/thymidine phosphorylase is up-regulated in breast cancer epithelium and endothelium. Br J Cancer 73:275-280

Frater-Schroder M, Risau W, Hallmann R, et al (1987). Tumour necrosis factor type alpha, a potent inhibitor of endothelial cell growth *in vitro*, is angiogenic *in vivo*. Proc Natl Acad Sci USA 84:5277-5281

Fujise N, Nanashim A, Taniguchi Y, et al (2000). Prognostic impact of cathepsin B and matrix metalloproteinase-9 in pulmonary adenocarcinomas by immunohistochemical study. Lung Cancer 27:19-26

Fukuda Y, Imoto M, Koyama Y, et al (1991). Immunohistochemical study on tissue inhibitors of metalloproteinases in normal and pathological human livers. Gastroenterol Jpn 26:37-41

Fukuyama Y, Mitsudomi T, Sugio K, et al (1997). K-ras and p53 are an independent unfavourable prognostic indicator in patients with non small cell lung cancer. Br J Cancer 75:1125-1130

Galateau-Salle FB, Luna RE, Horiba K et al (2000). Matrix metalloproteinases and tissue inhibitors of metalloproteinases in bronchial squamous preinvasive lesions. Hum Pathol 31:296-305

Garbisa S, Pozatti R, Muschel R, et al (1987). Secretion of type IV collagenolytic protease and metastatic phenotype: induction by transfection with c-Ha-ras but not with c-Ha-ras plus Ad2-E1a. Cancer Res 47:1523-1528

Gasparini G, Bevilacqua P, Bonoldi E, et al (1995). Predictive and prognostic markers in a series of patients with head and neck squamous cell invasive carcinoma treated with concurrent chemoradiation therapy. Clin Cancer Res 1:1375-1383

Gasparini G, Bonoldi E, Viale G, et al (1996). Prognostic and predictive value of tumour angiogenesis in ovarian carcinomas. Int J Cancer 69:205-211

Gately S, Twardowski P, Stack MS, et al (1997). The mechanism of cancer-mediated conversion of plasminogen to the angiogenesis inhibitor angiostatin. Proc Natl Acad Sci USA 94:10868-10872

Gavrilovic J, Hembry RM, Reynolds JJ, et al (1987). Tissue inhibitor of metalloproteinases (TIMP) regulates extracellular type 1 collagen degradation by chondrocytes and endothelial cells. J Cell Sci 87:357-362

Gazdar AF, Minna JD (2000). Angiogenesis and the multistage development of lung cancers. Clin Cancer Res 6:1611-1612

Gensersch E, Schuppan D, Lichtner RB (1996). Signaling by epidermal growth factor differentially affects integrin-mediated adhesion of tumor cells to extracellular matrix proteins. J Mol Med 74:609-616

Giatromanolaki A, Koukourakis M, O'Byrne K, et al (1996a). Prognostic value of angiogenesis in operable non-small cell lung cancer. J Pathol 179:80-88

Giatromanolaki A, Koukourakis M, O'Byrne K, et al, (1996b). Non small cell lung cancer: c-erbB-2 correlates with angiogenesis and poor prognosis. Anticancer Res 16:3819-3825

Giatromanolaki A, Koukourakis MI, Kakolyris S, et al (1998a). Focal expression of thymidine phosphorylase associates with CD31 positive lymphocytic aggregation and local neo-angiogenesis in non-small cell lung cancer. Anticancer Res 18:71-76

Giatromanolaki A, Koukourakis MI, Kakolyris S, et al (1998b). Vascular endothelial growth factor, wild-type p53 and angiogenesis in early operable non-small cell lung cancer. Clin Cancer Res 4:3017-3024

Giatromanolaki A, Koukourakis MI, Sivridis E, et al (2000). Coexpression of MUC1 glycoprotein with multiple angiogenic factors in non-small cell lung cancer suggests coactivation of angiogenic and migration pathways. Clin Cancer Res 6:1917-1920

Ginsberg RJ, Vokes EE, Raben A (1997). Cancer of the lung, section 2: Non-small cell lung cancer. In 'Cancer, Principles and Practice of Oncology'. DeVita VT, Hellman S & Rosenberg SA (Eds) p858-910. JB Lippincott, Philadelphia, PA

Gleave ME, Elhilal M, Fradet Y, et al (1998). Interferon gama-1b compared with placebo in metastatic renal-cell carcinoma. New Eng J Med 338:1265-1271

Goldberg GI, Strongin A, Collie IE, et al (1992). Interaction of 92-kDa type IV collagenases with the tissue inhibitor of metalloproteinases prevents dimerization, complex formation with interstitial collagenase, and activation of the proenzyme by stromelysin. J Biol Chem 267:4583-4591

Gonzalez-Avila G, Iturria C, Vadillo F, et al (1998). 72-kD (MMP-2) and 92-kD (MMP-9) type IV collagenase production and activity in different histological types of lung cancer cells. Pathobiology 66:5-16

Good DJ, Polverini PJ, Rastinejad F, et al (1990). A tumour suppressor-dependent inhibitor of angiogenesis is immunologically and functionally indistinguishable from a fragment of thrombospondin. Proc Natl Acad Sci USA 87:6624-6628

Goto F, Goto K, Weindel K, et al (1993). Synergistic effects of vascular endothelial growth factor and basic fibroblast growth factor on the proliferation and cord formation of bovine capillary endothelial cells within collagen gels. Lab Invest 69:508-517

Goulding H, Abdul Rashid NF, Robertson JF, et al (1995). Assessment of angiogenesis in breast carcinoma: an important factor in prognosis? Hum Pathol 26:1196-1200

Graeber TG, Peterson JF, Tsai M, et al (1994). Hypoxia induces accumulation of p53 protein, but activation of a G1-phase checkpoint by low-oxygen conditions is independent of p53 status. Mol Cell Biol 14:6264-6277

Graeber TG, Osmanian C, Jacks T, et al (1996). Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumors. Nature 379:88-91

Graziano C (1998). HER-2 breast assay, linked to Herceptin, wins FDA's okay. CAP Today 12:13-16

Griffiths L, Dachs GU, Bicknell R, et al (1997). The influence of oxygen tension and pH on the expression of platelet-derived endothelial cell growth factor/thymidine phosphorylase in human breast tumour cells grown in vitro and in vivo. Cancer Res 57:570-572

Grignon DJ, Sakr W, Toth M, et al (1996). High levels of tissue inhibitor of metalloproteinase-2 (TIMP-2) expression are associated with poor outcome in invasive bladder cancer. Cancer Res 56:1654-1659

Gualandris A, Urbinati C, Rusnati M, et al (1994). Interaction of high molecular weight basic fibroblast growth factor (bFGF) with endothelium: biological activity and intracellular fate of human recombinant Mr 24,000 bFGF. J Cell Physiol 161:149-159

Guddo F, Fontanini G, Reina C, et al (1999). The expression of basic fibroblast growth factor (bFGF) in tumour-associated stromal cells and vessels is inversely correlated with non-small cell lung cancer progression. Hum Pathol 30:788-794

Guidi AJ, Fischer L, Harris JA, et al (1994). Microvessel density and distribution in ductal carcinoma in situ of the breast. J Nat Cancer Inst 86:614-619

Guidi AJ, Abu-Jawdeh JG, Berse B, et al (1995). Vascular permeability factor (vascular endothelial growth factor) expression and angiogenesis in cervical neoplasia. J Natl Cancer Inst 87:506-516

Gum R, Lengyel E, Juarez J, et al (1996). Stimulation of 92-kDa gelatinase B promoter activity by ras is mitogen-activated protein kinase 1-independent and requires multiple transcription binding sites including closely spaced PEA3/ets and AP-1 sequences. J Biol Chem 271:10672-10680

Guo H, Zucker S, Gordon MK, et al (1997). Stimulation of matrix metalloproteinase production by recombinant matrix metalloproteinase inducer from transfected Chinese hamster ovary cells. J Biol Chem 1997;272:24-27.

Haeder M, Rotsch M, Belper G, et al (1988). Epidermal growth factor receptor expression in human lung cancer cell lines. Cancer Res 48:1132-1136

Hanahan D, Folkman J (1996). Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. Cell 86:353-364

Hanemaaijer R, Koolwijk P, le Clercq L, et al (1993). Regulation of matrix metalloproteinase expression in human vein and microvascular endothelial cells. Effects of tumor necrosis factor-alpha, interleukin-1 and phorbol ester. Biochem J 296:803-809

Hannekan A, Maher P, Baird A (1995). High affinity immunoreactive FGF receptors in the extracellular matrix of vascular endothelial cells- implications for the modulation of FGF-2. J Cell Biol 128:1221-1228

Harpole DH Jr, Herndon JH, Wolfe WG, et al (1995a). A prognostic model of recurrence and death in stage I non-small cell lung cancer utilizing presentation, and oncogene expression. Cancer Res 55:51-56

Harpole DH Jr, Marks JR, Richards WG, et al (1995b). Localized adenocarcinoma of the lung: oncogene expression of erbB-2 and p53 in 150 patients. Clin Cancer Res 1:659-664

Harpole DH Jr, Richards WG, Herndon JE, et al (1996). Angiogenesis and molecular biologic substaging in patients with stage I non-small cell lung cancer. Ann Thorac Surg 61:1470-1476

Harris AL (1997). Antiangiogenesis for cancer therapy. Lancet 349(suppl II):13-15

Hayakawa T, Yamashita K, Ohuchi E, et al (1994). Cell growth-promoting activity of tissue inhibitor of metalloproteinases-2 (TIMP-2). J Cell Sci 107:2373-2379

He C, Wilhelm SM, Pentland AP, et al (1989). Tissue co-operation in a proteolytic cascade activating human interstitial collagenase. Proc Natl Acad Sci 86:2632-2636

Heldin NE, Usuki K, Bergh J, et al (1993). Differential expression of platelet-derived endothelial cell growth factor/thymidine phosphorylase in human lung carcinoma cell lines. Br J Cancer 68:708-711

Hendler FJ, Ozanne B (1984). Human squamous cell lung cancers express increased epidermal growth factor receptors. J Clin Invest 74:647-651

Heppner KJ, Matrisian LM, Jensen RA, et al (1996). Expression of most matrix metalloproteinase family members in breast cancer represents a tumour induced host response. Am J Pathol 149:273-282

Herbst RS, Yano S, Kuniyasu H, et al (2000). Differential expression of E-cadherin and type IV collagenase genes predicts outcome in patients with stage I non-small cell lung cancer. Clin Cancer Res 6:790-797

Herron GS, Banda MJ, Clark EJ, et al (1986). Secretion of metalloproteinases by stimulated capillary endothelial cells. J Biol Chem 261:2814-2818

Hewitt RE, Leach IH, Powe DG, et al (1991). Distribution of collagenases and tissue inhibitors of metalloproteinases (TIMP) in colorectal tumours. Int J Cancer 49:666-672

Hickman JA, Potten CS, Merritt AJ, et al (1995). Apoptosis and cancer chemotherapy. Philos Trans R Soc London Ser B 345:83-89

Hida T, Leyton J, Makheja AN, et al (1998). Non-small cell lung cancer cycloxygenase activity and proliferation are inhibited by non-steroidal antiinflammatory drugs. Anticancer Res 18(2A):775-782

Hollingsworth HC, Kohn EC, Steimberg SM, et al (1995). Tumour angiogenesis in advanced stage ovarian carcinoma. Am J Pathol 147:33-41

Holm C, Geneitis D, McConville GS, et al (1996). Expression of PDGF, VEGF and their receptors in non-small-cell lung tumor lines. Int J Oncol 9:1077-1086

Holmgren L, O'Reilly MS, Folkman J (1995). Dormancy of micrometastases: balanced proliferation and apoptosis in the presence of angiogenesis suppression. Nature Med 1:149-153

Hori A, Sasada R, Matsunani E, et al (1991). Suppression of solid tumour growth by immunoneutralizing monoclonal antibody against human basic fibroblast growth factor. Cancer Res 51:6180-6184

Horio Y, Takahashi T, Kurioshi T, et al (1993). Prognostic significance of p53 mutations in primary resected non-small cell lung cancer. Cancer Res 53:1-4

Houck KA, Ferrara N, Winer J, et al (1991). The vascular endothelial cell growth factor family: identification of a fourth molecular species and characterization of alternative splicing of RNA. Mol Endocrinol 5:1806-1814

Howard EW, Bullen EC, Banda MJ (1991). Preferential inhibition of 72 and 92 kDa gelatinases by tissue inhibitor of metalloproteinases-2. J Biol Chem 266:13070-13075

Hua J, Muschel RJ (1996). Inhibition of matrixmetalloproteinase 9 expression by a ribozyme blocks metastasis in a rat sarcoma model system. Cancer Res 56:5279-5284

Huhtala P, Humphries MJ, McCarthy JB, et al (1995). Cooperative signalling by $\alpha_5\beta_1$ and $\alpha_4\beta_1$ integrins regulates metalloproteinase gene expression in fibroblasts adhering to fibronectin. J Cell Biol 129:867-879

Hurewitz AN, Zucker S, Mancuso P, et al (1992). Human pleural effusions are rich in matrix metalloproteinases. Chest 102:1808-1814

Hurskainen T, Soini Y, Tuuttila A et al (1996). Expression of the tissue metalloproteinase inhibitors TIMP-1 and TIMP-2 in malignant fibrous histiocytomas and dermatofibromas as studied by in situ hybridization and immunohistochemistry. Hum Pathol 27:42-49

Iizasa T, Fujisawa T, Suzuki M, et al (1999). Elevated levels of circulating plasma matrix metalloproteinase 9 in non-small cell lung cancer patients. Clin Cancer Res 5:149-153

Imai T, Koike K, Kubo T, et al (1991). Interleukin-6 supports human megakaryotype proliferation and differentiation in vitro. Blood 78:1959-1964

Imoto H, Osaki T, Taga S, et al (1998). Vascular endothelial growth factor expression in non-small-cell lung cancer: prognostic significance in squamous cell carcinoma. J Thorac Cardiovasc Surg 115:1007-1014

Imren S, Kohn DB, Schimar H, et al (1996). Over expression of tissue inhibitor of metalloproteinases-2 by retro-viral mediated gene transfer in vivo inhibits tumour growth and invasion. Cancer Res 56:2891-2895

Iruela-Arispe ML, Bornstein P, Sage H (1991). Thrombospondin exerts an antiangiogenic effect on tube formation by endothelial cells. Proc Natl Acad Sci USA 88:5026-5030

Ishikawa F, Miyazono K, Hellman U, et al (1989). Identification of angiogenic activity and the cloning and expression of platelet-derived endothelial cell growth factor. Nature 338:557-562

Ito A, Mukaiyama A, Itoh Y, et al (1996). Degradation of interleukin-1β by matrix metalloproteinases. J Biol Chem 271:14657-14660

Itoh T, Ikeda T, Gomi H, et al (1997). Unaltered secretion of B-amyloid precursor in gelatinase A (matrix metalloproteinase-2) –deficient mice. J Biol Chem 272:22389-22392

Itoh T, Tanioka M, Yoshida H, et al (1998). Reduced angiogenesis and tumour progression in gelatinase A-deficient mice. Cancer Res 58:1048-1051

Iwata H, Kobayashi S, Iwase H, et al (1996). Production of matrix metalloproteinases and tissue inhibitors of metalloproteinases in human breast carcinomas. Jap J Cancer Res 87:602-611

Jacobs TW, Gown AM, Yaziji H, et al (1999). Specificity of HercepTest in determining HER2/neu status of breast cancers using the United States Food and Drug Administration-approved scoring sytstem. J Clin Oncol 17:1983-7

Jacobs TW, Gown AM, Yaziji H, et al (2000). HER-2/neu protein expressionin breast cancer evaluated by immunohistochemistry. A study of interlaboratory agreement. Am J Clin Pathol 113:251-8

Jager R, Noll K, Pfluger KH, et al (1997). Differential expression and biological activity of the heparin-binding growth-associated molecule (HB-GAM) in lung cancer cell lines. Int J Cancer 73:537-543

Ji WR, Barrientos LG, Llinas M, et al (1998). Selective inhibition by kringle 5 of human plasminogen on endothelial cell migration, an important process in angiogenesis. Biochem Biophys Res Comm 247:414-419

Johnsen M, Lund LR, Romer J, et al (1998). Cancer invasion and tissue remodelling: common themes in proteolytic matrix degradation. Curr Opin Cell Biol 10:667-671

Johnson MD, Kim H-RC, Chesler L, et al (1994). Inhibition of angiogenesis by tissue inhibitor of metalloproteinases. J Cell Physiol 160:194-202

Jones JL, Royall JE, Walker RA (1996). E-cadherin relates to EGFR expression and lymph node metastasis in primary breast carcinoma. Br J Cancer 74:1237-1241

Kanayama H, Yokota K, Kurokawa Y, et al (1998). Prognostic values of matrix metalloproteinase-2 and tissue inhibitor of metalloproteinase-2 expression in bladder cancer. Cancer 82:1359-1366

Karameris A, Panagou P, Tsilalis T, et al (1997). Association of expression of metalloproteinases and their inhibitors with the metastatic potential of squamous-cell lung carcinomas. A molecular and immunohistochemical study. Am J Respir Crit Care Med 156:1930-1936

Karin M, Liu Z-G, Zandi E (1997). AP-1 function and regulation. Curr Opin Cell Biol 9:240-246

Kato Y, Nakayama Y, Umeda M, et al (1992). Induction of 103kDa gelatinase/type IV collagenase by acidic culture conditions in mouse metastatic melanoma cell lines. J Biol Chem 267:11424-11430

Katoh O, Tauchi H, Kawaishi K, et al (1995). Expression of the vascular endothelial growth factor (VEGF) receptor gene KDR, in haemopoietic cells and inhibitor effect of VEGF on apoptotic cell death caused by ionizing radiation. Cancer Res 55:5687-5692

Kawaguchi T, Yamamoto S, Kudoh S, et al (1997). Tumour angiogenesis as a major prognostic factor in stage I lung adenocarcinoma. Anticancer Res 17:3743-3746

Kawamata H, Kameyama S, Kawai K, et al (1995). Marked acceleration of the metastatic phenotype of a rat bladder carcinoma cell line by the expression of human gelatinase A. Int J Cancer 63:568-575

Kawano N, Osawa H, Ito T, et al (1997). Expression of gelatinase A, tissue inhibitor of metalloproteinases-2, matrilysin, and trypsin(ogen) in lung neoplasms: an histochemical study. Hum Pathol 28:613-622

Keith RL, Miller YE, Gemmill RM et al (2000). Angiogenic squamous dysplasia in bronchi of individuals at high risk for lung cancer. Clin Cancer Res 6:1616-1625

Keiser A, Weich HA, Brandner G, et al (1994). Mutant p53 potentiates protein kinase C induction of vascular endothelial growth factor expression. Oncogene 9:964-969

Kern JA, Schwartz DA, Norberg JE, et al (1990). p185*neu* expression in human lung adenocarcinomas predicts shortened survival. Cancer Res 50:5184-5191

Khokha R, Waterhouse P, Yagel S, et al (1989). Antisense RNA-induced reduction in metalloproteinase inhibitor causes mouse 3T3 cells to become tumorigenic. Science 243:947-950

Khokha R, Zimmer MJ, Wilson SM, et al (1992). Up-regulation of TIMP-1 expression in B16-F10 melanoma cells suppresses their metastatic ability in chick embryo. Clin Exp Metastasis 32:365-370

Klein S, Giancotti FG, Presta M, et al (1993). Basic fibroblast growth factor modulates integrin expression in microvascular endothelial cells. Mol Biol Cell 4:973-982

Kleiner DJ, Stetler-Stevenson WG (1993). Structural biochemistry and activation of matrix metalloproteinases. Curr Opin Cell Biol 5:891-897

Kobayashi N, Ishii M, Ueno Y, et al (1999). Co-expression of Bcl-2 protein and vascular endothelial growth factor in hepatocellular carcinomas treated by chemoembolization. Liver 19:25-31

Kodate M, Kasai T, Hashimoto H, et al (1997). Expression of matrix metalloproteinase (gelatinase) in T1 adenocarcinoma of the lung. Pathol Int 47:461-469

Kondapaka SB, Fridman R, Reddy KB (1997). Epidermal growth factor and amphiregulin up-regulate matrix metalloproteinase-9 (MMP-9) in human breast cancer cells. Int J Cancer 70:722-726

Kondo S, Asano M, Suzuki H (1993). Significance of vascular endothelial growth factor/vascular permeability factor for solid tumour growth, and its inhibition by the antibody. Biochem Biophys Res Commun 190:1234-1241

Kondo S, Asano M, Matsuo K, et al (1994). Vascular endothelial growth factor/vascular permeability factor is detectable in the sera of tumor-bearing mice and cancer patients. Biochim Biophys Acta 1221:211-214

Koomagi R, Volm M (1998). Tissue-factor expression in human non-small-cell lung carcinoma measured by immunohistochemistry: correlation between tissue factor and angiogenesis. Int J Cancer 79:19-22

Koop S, Khokha R, Schmidt EC, et al (1994). Overexpression of metalloproteinase inhibitor in B16 F10 cells does not affect extravasation but reduces tumour growth. Cancer Res 54:4791-4797

Kostyleva OI, Gerstein ES, Dykhno AI, et al (1999). EGF-like peptides and their receptors as prognostic factors for the survival of patients with non-small-cell lung cancer. Vopr Onkol 45:617-622

Koukourakis MI, Giatromanolaki A, O'Byrne KJ, et al (1997a). Platelet-derived endothelial cell growth factor expression correlates with tumour angiogenesis and prognosis in non-small-cell lung cancer. Br J Cancer 75:477-481

Koukourakis MI, Giatromanolaki A, O'Byrne KJ, et al (1997b). Potential role of bcl-2 as a suppressor of tumour angiogenesis in non-small cell lung cancer. Int J Cancer 74:565-570

Koukourakis MI, Giatromanolaki A, Kakolyris S, et al (1998). Different patterns of stromal and cancer cell thymidine phosphorylase reactivity in non-small-cell lung cancer: impact on tumour neoangiogenesis and survival. Br J Cancer 77:1696-1703

Koukourakis MI, Giatromanolaki A, O'Byrne KJ, et al (1999). bcl-2 and c-erbB-2 proteins are involved in the regulation of VEGF and of thymidine phosphorylase angiogenic activity in non-small cell lung cancer. Clin Exp Metastasis 17:545-554

Koukourakis MI, Giatromanolaki A, Thorpe PE, et al (2000). Vascular endothelial growth factor/KDR activated microvessel density versus CD31 standard microvesel density in non-small cell lung cancer. Cancer Res 60:3088-3095

Kusaka M, Sudo K, Matsutani E, et al (1994). Cytostatic inhibition of endothelial cell growth by the angiogenic inhibitor TNP-470 (AGM-1470). Br J Cancer 69:212-216

Kuwabara K, Ogawa S, Matsumoto M, et al (1995). Hypoxia-mediated induction of acid/basic fibroblast growth factor and platelet-derived growth factor in mononuclear phagocytes stimulates growth of hypoxic endothelial cells. Proc Natl Acad Sci USA 92:4606-4610

Lamoreaux WJ, Fitzgerald MEC, Reiner A, et al (1998). Vascular endothelial growth factor increases release of gelatinase A and decreases release of tissue inhibitor of metalloproteinases by microvascular endothelial cells in vitro. Microvascular Res 55:29-42

Laudanski J, Chyczewski L, Niklinska WE et al (1999). Expression of bcl-2 protein in non-small cell lung cancer: correlation with clinicopathology and patient survival. Neoplasma 46:25-30

Leco KJ, Khokha R, Pavloff N, et al (1994). Tissue inhibitor of metalloproteinases-3 (TIMP-3) is an extracellular matrix associated protein with a distinctive pattern of expression in mouse cells and tissues. J Biol Chem 269:9352-9360

Lee JS, Yoon A, Kalapurakal SK, et al (1995). Expression of p53 oncoprotein in non-small cell lung cancer: a favourable prognostic indicator. J Clin Oncol 13:1893-1903

Leek RD, Lewis CE, Whitehouse R, et al (1996). Association of macrophage infiltration with angiogenesis and prognosis in invasive breast carcinoma. Cancer Res 56:4625-4629

Leevers SJ, Vanhaesebroeck B, Waterfield MD (1999). Signalling through phosphoinositide 3-kinases: the lipids take centre stage. Curr Opin Cell Biol 11:219-225

Lei W, Mayotte JE, Levitt ML (1999). Enhancement of chemosensitivity and programmed cell death by tyrosine kinase inhibitors correlates with EGFR expression in non-small cell lung cancer. Anticancer Res 19:221-228

Levi E, Fridman R, Miao H-Q, et al (1996). Matrix metalloproteinase 2 releases active soluble ectodomain of fibroblast growth factor receptor 1. Proc Natl Acad Sci USA 93:7069-7074

Levin WJ, Press MF, Gaynor RB, et al (1995). Expression patterns of immediate early transcription factors in human non-small cell lung cancer. Oncogene 11:1261-1269

Levy AP, Levy NS, Goldberg MA (1996). Post-transcriptional regulation of vascular endothelial growth factor by hypoxia. J Biol Chem 271:2746-2753

Lewis TS, Shapiro PS, Ahn NG (1998). Signal transduction through MAP kinase cascades. Adv Cancer Res 74:49-129

Li J, Perella MA, Tsai J-C, et al (1995). Induction of vascular endothelial growth factor gene expression by interleukin-1β in rat aortic smooth muscle cells. J Biol Chem 1995;270:308-312

Liotta LA, Rao N, Barsky SH (1993). Tumour invasion and the extracellular matrix. Lab Invest 49:636-649

Llorens A, Rodrigo I, Lopez-Barcons L, et al (1998). Down-regulation of E-cadherin in mouse skin carcinoma cells enhances a migratory and invasive phenotype linked to matrix metalloproteinase-9 gelatinase expression. Lab Invest 78:1131-1142

Lo CJ, Cryer HG, Fu M, et al (1998). Regulation of macrophage eicosanoid generation is dependent on nuclear factor kappaB. J Trauma 45:19-23

Lotan R (1991). Retinoids as modulators of tumor cells invasion and metastasis. Semin Cancer Biol 2:197-208

Lotem J, Sachs L (1993). Regulation by bcl-2, c-myc, and p53 of susceptibility to induction of apoptosis by heat shock and cancer chemotherapy compounds in differentiation-competent and defective myeloid leukaemia cells. Cell Growth Different 4:41-47

Luscinskas FW, Lawler J (1994). Integrins as dynamic regulators of vascular function. Fed Am Soc Exp Biol J 8:929-938

Macchiarini P, Fontanini G, Hardin JM, et al (1992). Relation of neovascularisation to metastasis of non-small-cell lung cancer. Lancet 340:145-146

Macaulay VM, O'Byrne KJ, Saunders MP, et al (1999). Phase I study of intrapleural batimastat (BB-94), a matrix metalloproteinase inhibitor, in the treatment of malignant pleural effusions. Clin Cancer Res 5:513-520

Maeda K, Chung Y, Ogawa Y, et al (1996). Prognostic value of vascular endothelial growth factor expression in gastric carcinoma. Cancer 77:858-863

Maione TE, Gray GS, Petro J, et al (1990). Inhibition of angiogenesis by recombinant platelet factor-4 and related peptides. Science 247:77-79

Majima M, Isono M, Ikeda Y, et al (1997). Significant roles of inducible cyclooxygenase (COX)-2 in angiogenesis in rat sponge implants. Jpn J Pharmacol 75:105-114

Maniwa Y, Okada M, Ishii N, et al (1998). Vascular endothelial growth factor increased by pulmonary surgery accalerates the growth of micrometastases in metastatic lung cancer. Chest 114:1668-1675

Mann JS, Kindy MS, Edwards DR, et al (1991). Hormonal regulation of matrix metalloproteinase inhibitors in rat granulosa cells and ovaries. Endocrinology 128:1825-1832

Martin L, Green B, Renshaw C, et al (1997a). Examining the technique of angiogenesis assessment in invasive breast cancer. Br J Cancer 76:1046-1054

Martin L, Holcombe C, Green B, et al (1997b). Is a histological section representative of whole tumour vascularity in breast cancer? Br J Cancer 76:40-43

Matsuyama K, Chiba Y, Sasaki M, et al (1998). Tumour angiogenesis as a prognostic marker in operable non-small cell lung cancer. Ann Thorac Surg 65:1405-1409

Mattern J, Koomagi R, Volm M (1995). Vascular endothelial growth factor expression and angiogenesis in non-small cell lung carcinomas. Int J Oncol 6:1059-1062

Mattern J, Koomagi R, Volm M (1996). Association of vascular endothelial growth factor expression with intratumoral microvessel density and tumour cell proliferation in human epidermoid lung carcinoma. Br J Cancer 73:931-934

Mattern J, Koomagi R, Volm M (1997). Coexpression of VEGF and bFGF in human epidermoid lung carcinoma is associated with increased vessel density. Anticancer Res 17:2249-2252

Mauceri HJ, Hanna NN, Beckett MA, et al (1998). Combined effects of angiostatin and ionizing radiation in antitumour therapy. Nature 394:287-291

McCawley LJ, O'Brien P, Hudson LG (1998). Epidermal growth factor (EGF)- and scatter factor/hepatocyte growth factor (SF/HGF)-mediated keratinocyte migration is coincident with induction of matrix metalloproteinase (MMP)-9. J Cell Physiol 176:255-265

McCulloch P, Choy A, Martin L (1995). Association between tumour angiogenesis and tumour cell shedding into effluent venous blood during breast cancer surgery. Lancet 346:1334-1335

McDonnell TJ, Troncoso P, Brisbay SM, et al (1992). Expression of the protooncogene bcl-2 in the prostate and its association with emergence of androgen-independent prostate cancer. Cancer Res 52:6940-6944

140

McLaren R, Kuzu I, Dunnil M, et al (1992). The relationship of p53 immunostaining to survival in carcinoma of the lung. Br J Cancer 66:735-738

Meade-Tollin LC, Boukamp P, Fusenig NE, et al (1998). Differential expression of matrix metalloproteinases in activated c-*ras*Ha-transfected immortalized human keratinocytes. Br J Cancer 77:724-730

Mestre JR, Chan G, Zhang F, et al (1997). Inhibition of cyclooxygenase-2 expression. An approach to preventing head and neck cancer. Ann N Y Acad Sci 889:62-71

Mignatti P, Tsubio R, Robbins E, et al (1989). In vitro angiogenesis on the human amniotic membrane: requirement for basic fibroblast growth factor-induced proteinases. J Cell Biol 108:671-678

Mignatti P, Mazzieri R, Rifkin DB (1991). Expression of the urokinase receptor in vascular endothelial cells is stimulated by the basic fibroblast growth factor. J Cell Biol 113:1193-1201

Millauer B, Shawver LK, Plate KH, et al (1994). Glioblastoma growth inhibited *in vivo* by a dominant-negative Flk-1 mutant. Nature 367:576-579

Minn AJ, Swain RE, Ma A, et al (1998). Recent progress on the regulation of apoptosis by Bcl-2 family members. Advances in Immunology 70:245-279

Miyamoto S, Teramoto H, Gutkind JS, et al (1996). Integrins can collaborate with growth factors for phosphorylation of receptor tyrosine kinases and MAP kinase activation: roles of integrin aggregation and occupancy of receptors. J Cell Biol 135:1633-1642

Miyashita T, Reed JC (1992). Bcl-2 gene transfer increases relative resistance of S49.1 and WEH17.2 lymphoid cells to cell death and DNA fragmentation induced by glucocorticoids and multiple chemotherapeutic regimes. Cancer Res 52:5407-5411

Miyashita T, Krajewski S, Krajewski M, et al (1994). Tumour suppressor p53 is a regulator of bcl-2 and bax gene expression *in vitro* and *in vivo*. Oncogene 9:1799-1805

Modjtahedi H, Styles J, Dean C (1993). The growth response of human tumor cell lines expressing the EGF receptor to treatment with EGF and/or Mabs that block ligand binding. Int J Oncol 3:237-243

Moghaddam A, Bicknell R (1992). Expression of platelet-derived endothelial cell growth factor in *Escherichia coli* and confirmation of its thymidine phosphorylase activity. Biochemistry 31:12141-12146

Moghaddam A, Zhang HT, Fan TPD, et al (1995).Thymidine phosphorylase is angiogenic and promotes tumour growth. Proc Natl Acad Sci USA 92:998-1002

Mohle R, Green D, Moore MA, et al (1997). Constitutive production and thrombin-induced release of vascular endothelial growth factor by human megakaryocytes and platelets. Proc Natl Acad Sci USA 94:663-668

Molina MA, Sitja-Arnau M, Lemoine MG, et al (1999). Increased cyclooxygenase-2 expression in human pancreatic carcinomas and cell lines: growth inhibition by non-steroidal anti-inflammatory drugs. Cancer Res 59:4356-4362

Monteagudo C, Merino MJ, San-Juan J, et al (1990). Immunohistochemical distribution of type IV collagenase in normal, benign and malignant breast tissue. Am J Pathol 136:585-592

Montesano R, Vissalli J-D, Baird A, et al (1986). Basic fibroblast growth factor induces angiogenesis in vitro. Proc Natl Acad Sci USA 83:7297-73

Montgomery AM, Mueller BM, Reisfield RA, et al (1994). Effect of tissue inhibitor of the matrix metalloproteinases-2 expression on the growth and spontaneous metastasis of a human melanoma cell line. Cancer Res 54:5467-5473

Morris VL, Koop S, MacDonald IC, et al (1994). Mammary carcinoma cell lines of high and low metastatic potential differ not in extravasation but in subsequent migration and growth. Clin Exp Metastasis 12:357-367

Moses MA, Langer R (1991). A metalloproteinase inhibitor as an inhibitor of neovascularization. J Cell Biochem 47:230-235

Moss ML, Jin S-LC, Milla ME, et al (1997). Cloning of a disintegrin metalloproteinase that processes precursor tumour-necrosis factor-α. Nature 385:733-736

Mountain CF (1997). Revisions in the international system for staging lung cancer. Chest 111:1711-1717

Mukhopadhyay D, Tsiokas L, Sukhatme VP (1995). Wild-type p53 and v-Src exert opposing influences on human vacular endothelial growth factor gene expression. Cancer Res 55:6161-6165

Muller D, Breathnach R, Engelmann A, et al (1991). Expression of collagenase-related metalloproteinase genes in human lung or head and neck tumours. Int J Cancer 48:550-561

Murphy AN, Unsworth EJ, Stetler-Stevenson WG (1993). Tissue-inhibitor of metalloproteinases-2 inhibits bFGF induced human microvascular endothelial cell proliferation. J Cell Physiol 157:351-358

Nagakawa H, Yagihashi S (1994). Expression of type IV collagen and its degrading enzymes in squamous cell carcinoma of the lung. Jap J Cancer Res 85:934-938

Nakamura H, Ueno H, Yamashita K, et al (1999). Enhanced production and activation of progelatinase A mediated by membrane-type 1 matrix metalloproteinase in human papillary thyroid carcinomas. Cancer Res 59:467-473

Naldini L, Tamagnone L, Vigna E, et al (1992). Extracellular proteolytic cleavage by urokinase is required for the activation of hepatocyte growth factor/scatter factor. EMBO J 11:4825-4833

Naumovski L, Cleary ML (1996). The p53-binding protein 53BP2 also interacts with Bcl-2 and impedes cell cycle progression at G2/M. Mol Cell Biol 16:3884-3892

Nawrocki B, Polette M, Marchand V, et al (1997). Expression of matrix metalloproteinases and their inhibitors in human bronchopulmonary carcinomas: quantitative and morphological analyses. Int J Cancer 72:556-564

Newell KJ, Witty JP, Rodgers WH, et al (1994). Expression and localization of matrix-degrading metalloproteinase during colorectal tumorigenesis. Mol Carcinog 10:199-206

Nguyen M, Strubel NA, Bischoff J (1993). A role for sialyl Lewis-x/a glycoconjugates in capillary morphogenesis. Nature 365:267-269

Nishio M, Koshikawa T, Kuroishi T, et al (1996). Prognostic significance of abnormal p53 accumulation in primary, resected non-small cell lung cancers. J Clin Oncol 14:497-502

Nomura H, Sato H, Seiki M, et al (1995). Expression of membrane-type matrix metalloproteinase in human gastric carcinomas. Cancer Res 55:3263-3266

Nomura H, Fujimoto N, Seiki M, et al (1996). Enhanced production of matrix metalloproteinases and activation of matrix metalloproteinase 2 (gelatinase A) in human gastric carcinomas. Int J Cancer 69:9-16

Nor JE, Christensen J, Mooney DJ, et al (1999). Vascular endothelial growth factor (VEGF)-mediated angiogenesis is associated with enhanced endothelial cell survival and induction of Bcl-2 expression. Am J Pathol 154:375-384154:375-384

Nor JE, Mitra RS, Sutorik MM, et al (2000). Thrombospondin-1 induces endothelial cell apoptosis and inhibits angiogenesis by activating the caspase death pathway. J Vasc Res 37:209-218

142

Nunez G, London L, Hockenberry D, et al (1990). Deregulated bcl-2 gene expression selectively prolongs survival of growth factor-deprived haemopoeitic cell lines. J Immunol 144:3602-3610

O'Brien TS, Smith K, Cranston D, et al (1995). Urinary basic fibroblast growth factor in patients with bladder cancer and benign prostatic hypertrophy. Br J Urol 76:311-314

O'Byrne KJ, Dobbs N, Propper D, et al (1999). Vascular endothelial growth factor, platelet counts, and prognosis in renal cancer. Lancet 353:1494-1495

O'Byrne KJ, Dobbs N, Harris AL (2000a). Serum vascular endothelial growth factor load and interleukin-6 in cancer patients. Br J Cancer 82:1895

O'Byrne KJ, Dalgleish AG, Browning MJ, et al (2000b). The relationship between angiogenesis and the immune response in carcinogenesis and progression of malignant disease. Eur J Cancer 36:151-169

O'Byrne KJ, Koukourakis MI, Giatromanolaki A, et al (2000c). Vascular endothelial growth factor, platelet-derived endothelial cell growth factor and angiogenesis in non-small cell lung cancer. Br J Cancer 82:1427-1432

O-charoenrat P, Modjtahedi H, Rhys-Evans P, et al (2000a). Epidermal growth factor-like ligands differentially up-regulate matrix metalloproteinase 9 in head and neck squamous carcinoma cells. Cancer Res 60:1121-1128

O-charoenrat P, Rhys-Evans P, Modjtahedi H, et al (2000b). Overexpression of epidermal growth factor receptor in human head and neck squamous carcinoma cell lines correlates with matrix metalloproteinase-9 expression and in vitro invasion. Int J Cancer 86:307-317

Office for National Statistics (1998). Data provided on request.

Ohori P, Yousem SA, Griffin J, et al (1992). Comparison of extracellular matrix antigens in subtypes of bronchioalveolar carcinoma and conventional pulmonary adenocarcinoma. Immunohistochemical study. Am J Surg Pathol 16:675-686

Ohsaki Y, Toyoshima E, Fujiuchi S, et al (1996). Bcl-2 and p53 protein expression in non-small cell lung cancer: correlation with survival time. Clin Cancer Res 2:915-920

Ohsaki Y, Tanno S, Fujita Y, et al (2000). Epidermal growth factor receptor expression correlates with poor prognosis in non-small cell lung cancer patients with p53 overexpression. Oncol Rep 7:603-607

Ohta Y, Endo Y, Tanaka M, et al (1996). Significance of vascular endothelial growth factor messenger RNA expression in primary lung cancer. Clin Cancer Res 2:1411-1416

Ohta Y, Watanabe Y, Murakami S, et al (1997). Vascular endothelial growth factor and lymph node metastasis in primary lung cancer. Br J Cancer 76:1041-1045

O'Hagan RC, Tozer RG, Symons M, et al (1996). The activity of the Ets transcription factor PEA3 is regulated by two distinct MAPK cascades. Oncogene 13:1323-1333

Olson TA, Mohanraj D, Carson LE, et al (1994). Vascular permeability factor gene expression in normal and neoplastic ovaries. Cancer Res 54:276-280

O'Neill AJ, Staunton MJ, Gaffney EF (1996). Apoptosis ocurs independently of bcl-2 and p53 over-expression in non-small cell lung cancer. Histopathology 29:45-50

O'Reilly MS, Holmgren L, Shing Y, et al (1994). Angiostatin: a novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. Cell 79:315-328

O'Reilly MS, Holmgren L, Chen C, et al (1996). Angiostatin induces and sustains dormancy of primary tumours in mice. Nat Med 2:689-692

O'Reilly MS, Boehm T, Shing Y, et al (1997). Endostatin: an endogenous inhibitor in angiogenesis and tumour growth. Cell 88:277-285

Oshika Y, Nakamura M, Tokunaga T, et al (1998). Expression of cell-associated isoform of vascular endothelial growth factor 189 and its prognostic relevance in non-small cell lung cancer. Int J Oncol 12: 541 – 544

Paesmans M, Sculier JP, Libert P, et al (1995). Prognostic factors for survival in advanced non-small-cell lung cancer: univariate and multivariate analyses including recursive and amalgamation algorithms in 1,052 patients. The European Lung Cancer Working Party. J Clin Oncol 13:1221-30

Passlick B, Izbicki JR, Haussinger K, et al (1995). Immunohistochemical detection of p53 is not associated with a poor prognosis in non-small cell lung cancer. J Thor Cardiovasc Surg 109:1205-1211

Pastorino U, Adreola S, Tagliube E, et al (1997). Immunocytochemical markers in stage I lung cancer: relevance to prognosis. J Clin Oncol 15:2858-2865

Patterson A, Zhang HT, Fan TPD, et al (1995). Increased sensitivity to the pro-drug 5'-deoxy-5-fluorouridine and modulation of 5'-fluoro-2'-deoxyuridine sensitivity in MCF-7 cell transfected with thymidine phosphorylase. Br J Cancer 72:669-675

Patterson BC, Sang QA (1997). Angiostatin-converting enzyme activities of human matrilysin (MMP-7) and gelatinase B/type IV collagenase (MMP-9). J Biol Chem 272:28823-28825

Pauly JL, Schuller MG, Zelcer AA, et al (1977). Identification and comparative analysis of thymidine phosphorylase in the plasma of healthy subjects and cancer patients. J Natl Cancer Inst 58:1587-1590

Pedersen LM, Milman N (1996). Prognostic significance of thrombocytosis in patients with primary lung cancer. Eur Resp J 9:1826-1830

Pepper MS, Belin D, Montesano R, et al (1990). Transforming growth factor-beta 1 modulates basic fibroblast growth factor-induced proteolytic and angiogenic properties of endothelial cells in vitro. J Cell Biol 111:743-755

Pepper MS, Ferrara N, Orci L, et al (1992a). Vascular endothelial growth factor (VEGF) induces plasminogen activators and plasminogen activator inhibitor type 1 in microvascular endothelial cells. J Cell Physiol 181:902-908

Pepper MS, Ferrara N, Orci L, et al (1992b). Potent synergism between vascular endothelial growth factor and basic fibroblast growth factor in the induction of angiogenesis *in vitro*. Biochem Biophys Res Comm 189:824-831

Petit AM, Rak J, Hung MC, et al (1997). Neutralizing antibodies against epidermal growth factor and ErbB-2/neu receptor tyrosine kinases down-regulate vascular endothelial growth factor production by tumor cells in vitro and in vivo: angiogenic implications for signal transduction therapy of solid tumors. Am J Pathol 151:1523-1530

Pezzella F, Turley H, Kuzu I, et al (1993). Bcl-2 protein in non-small cell lung carcinoma. N Eng J Med 329:690-694

Pezzella F, Pastorino U, Tagliabue E, et al (1997). Non-small cell lung carcinoma tumor growth without morphological evidence of neo-angiogenesis. Am J Pathol 151:1417-1423

Pfeiffer P, Claussen PP, Andersen K, et al (1996). Lack of prognostic significance of epidermal growth factor receptor and the oncoprotein p185^{HER-2} in patients with systematically untreated non-small-cell lung cancer: an immunohistochemical study on cryosections. Br J Cancer 74:86-91

Pinedo HM, Verheul HMW, D'Amato RJD, et al (1998). Involvement of platelets in tumour angiogenesis? Lancet 352:1775-1777

144

Polette M, Gilbert N, Stas I, et al (1994). Gelatinase A expression and localization in human breast cancers. An ISH study and immunohistochemical detection using confocal microscopy. Virchows Arch 426:29-35

Polette M, Nawrocki B, Gilles C, et al (1996). MT-MMP expression and localization in human lung and breast cancers. Virchows Arch 428:29-35

Polverini PJ, Leibovich SJ (1984). Induction of neovascularization in vivo and endothelial cell proliferation in vitro by tumour associated macrophages. Lab Invest 51:635-642

Porter AC, Vaillancourt RR (1998). Tyrosine kinase receptor-activated signal transduction pathways which lead to oncogenesis. Oncogene 17:1343-1352

Powe DG, Brough JL, Carter GI, et al (1997). TIMP-3 mRNA expression is regionally increased in moderately and poorly differentiated colorectal adenocarcinoma. Br J Cancer 75:1678-1683

Powell WC, Knox JD, Navre M, et al (1993). Expression of the metalloproteinase matrilysin in DU-145 cells increases their invasive potential in severe combined immunodeficient mice. Cancer Res 53:417-422

Press MF, Bernstein L, Thomas PA, et al (1997). HER-2/neu gen amplification characterized by fluorescence in situ hybridization: poor prognosis in node-negative breast carcinomas. J Clin Oncol 15:2894-904

Pyke C, Ralfkaier E, Tryggvason K, et al (1993). Messenger RNA for two type IV collagenases is located in stromal cells in human colon cancer. Am J Pathol 142:359-365

Quinlan DC, Davidson AG, Summers CL, et al (1992). Accumulation of p53 correlates with poor prognosis in human lung cancer. Cancer Res 52:4828-4831

Rasmussen HS, McCann PP (1997). Matrix metalloproteinase inhibition as a novel anticancer strategy: a review with special focus on batimastat and marimastat. Pharmacol Ther 75:69-75

Ray JM, Stetler-Stevenson WG (1994). The role of matrix metalloproteinases and their inhibitors in tumour invasion, metastasis and angiogenesis. Eur Resp J 7:2062-2072

Reddy KB, Kreuger JS, Kondapaka S.B, et al (1999). Mitogen-activated protein kinase (MAPK) regulates the expression of progelatinase B (MMP-9) in breast epithelial cells. Int J Cancer 82:268-273

Ree AH, Florenes VA, Berg JP, et al (1997). High levels of mRNA for tissue inhibitors of metalloproteinases (TIMP-1 and TIMP-2) in primary breast carcinomas are associated with development of metastases. Clin Cancer Res 3:1623-1628

Relf M, LeJeune S, Scott PAE, et al (1997). Expression of the angiogenic factors vascular endothelial growth factor, acidic and basic fibroblast growth factor, tumour growth factor α-1, platelet-derived endothelial cell growth factor, placenta growth factor, and plieotrophin in human primary breast cancer and its relation to angiogenesis. Cancer Res 57:963-969

Retera JM, Leers MP, Sulzer MA, et al (1998). The expression of beta-catenin in non-small-cell lung cancer: a clinicopathological study. J Clin Pathol 51:891-894

Rhodes T, Twentyman PR (1992). A study of ethacrynic acid as a potential modifier of melphalan and cisplatin sensitivity in human lung cancer parental and drug-resistant cell lines. Br J Cancer 65:684-690

Roche PC, Ingle JN (1999). Increased HER2 with US Food and Drug Administration-approved antibody {letter}. J Clin Oncol 17:434

Rosenthal EL, Johnson TM, Allen ED, et al (1998). Role of the plasminogen activator and matrix metalloproteinase systems in epidermal growth factor- and scatter factor-stimulated invasion of carcinoma cells. Cancer Res 58:5221-30

145

Rusch V, Klimstra D, Venkatraman E, et al (1997). Overexpression of the epidermal growth factor receptor and its ligand transforming growth factor alpha is frequent in resectable non-small cell cancer but does not predict tumor progression. Clin Cancer Res 3:515-522

Ryan JJ, Prochownik E, Gottlieb CA, et al (1994). C-myc and bcl-2 modulate p53 function by altering p53 subcellular trafficking during the cell cycle. Proc Natl Acad Sci USA 91:5878-5882

Saclarides TJ, Speziale NJ, Drab E, et al (1994). Tumor angiogenesis and rectal carcinoma. Dis Colon Rectum 37:921-926

Saegusa M, Takano Y, Hashimura M, et al (1995). The possible role of bcl-2 expression in the progression of tumours of the uterine cervix. Cancer 76:2297-2303

Sakuragi N, Ohkouchi T, Hareyama H, et al (1998). Bcl-2 expression and prognosis in patients with endometrial carcinoma. Int J Cancer 79:153-158

Salgado R, Vermeulen PB, Benoy I, et al (1999). Platelet number and interleukin-6 correlate with VEGF but not bFGF serum levels of advanced cancer patients. Br J Cancer 80:892-897

Salgado R, Benoy I, Weytjens R, et al (2000). Serum vascular endothelial growth factor load and interleukin-6 in cancer patients- reply. Br J Cancer 82:1896

Sato H, Kida Y, Mai M, et al (1992). Expression of genes encoding type IV collagen-degrading metalloproteinases and tissue inhibitors of metalloproteinases in various human tumor cells. Oncogene 7:77-83

Sato H, Takino T, Okada Y, et al (1994). A matrix metalloproteinase expressed on the surface of invasive tumour cells. Nature 370:61-65

Sato H, Kinoshita T, Yakino T, et al (1996). Activation of a recombinant membrane type 1-matrix metalloproteinase (MT1-MMP) by furin and its interaction with tissue inhibitor of metalloproteinases (TIMP)-2. FEBS Lett 393:101-104

Sato T, Nakajima H, Fujio K, et al (1997). Enhancement of prostaglandin E2 production by epidermal growth factor requires the coordinate activation of cytosolic phospholipase A2 and cyclooxygenase 2 in human squamous carcinoma A431 cells. Prostaglandins 53:355-369

Schnaper HW, Grant DS, Stetler-Stevenson WG, et al (1993). Type IV collagenase(s) and TIMPs modulate endothelial cell morphogenesis in vitro. J Cell Physiol 156:235-246

Schor AM, Pazoki S, Morris J, et al (1998). Heterogeneity in microvessel density in lung tumours: comparison with normal bronchus. Br J Cancer 77:946-951

Schultz RM, Silberman S, Persky B, et al (1988). Inhibition of recombinant tissue inhibitor of metalloproteinases of human amniotic invasion and lung colonisation by murine B16-F10 melanoma cells. Cancer Res 48:5539-5545

Schwartz EL, Baptiste N, O'Connor CJ, et al (1994). Potentiation of the antitumor activity of 5-fluorouracil in colon carcinoma cells by the combination of interferon and deoxyribonucleosides results from complementary effects on thymidine phosphorylase. Cancer Res 54:1472-1478

Schweiki D, Itin A, Soffer D, et al (1992). Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. Nature 359:843-845

Sekido Y, Fong KM, Minna JD (1998). Progress in understanding the molecular pathogenesis of human lung cancer. Biochim Biophys Acta F21-F59

Semenza GL (1996). Transcriptional regulation by hypoxia-inducible factor 1- molecular mechanisms in oxygen homeostasis. Trends Cardiovasc Med 6:151-157

Senger DR, Brown LF, Claffey KP, et al (1994). Vascular permeability factor, tumour angiogenesis and stroma generation. Invasion Metastasis 14:385-394

Shaheen RM, Davis DW, Liu W, et al (1999). Antiangiogenic therapy targeting the tyrosine kinase receptor for vascular endothelial growth factor inhibits the growth of colon cancer liver metastasis and induces tumor and endothelial cell apoptosis. Cancer Res 59:5412-5416

Shalaby F, Rossant J, Yamaguchi TP, et al (1995). Failure of blood-island formation and vasculogenesis in Flk-1 deficient mice. Nature 376:62-66

Shibanuma H, Hirano T, Tsuji K, et al (1998). Influence of E-cadherin dysfunction upon local invasion and metastasis in non-small cell lung cancer. Lung Cancer 22:85-95

Siegfried JM, Weissfeld LA, Luketich JD, et al (1998). The clinical significance of hepatocyte growth factor for non-small cell lung cancer. Ann Thor Surg 66:1915-1918

Sikora J, Slodkowska J, Radomyski A, et al (1997). Immunohistochemical evaluation of tumour angiogenesis in adenocarcinoma and squamous cell carcinoma of the lung. Rocz Akad Med Bialymst 42S1:271-279

Silvestrini R, Benini E, Veneroni S, et al (1996). p53 and bcl-2 expression correlates with clinical outcome in a series of node-positive breast cancer patients. J Clin Oncol 14:1604-1610

Simon C, Goepfert H, Boyd D (1998). Inhibition of the p38 mitogen-activated protein kinase by SB 203580 blocks PMA-induced Mr 92,000 type IV collagenase secretion and in vitro invasion. Cancer Res 58:1135-1139

Skobe M, Rockwell P, Goldstein N, et al (1997). Halting angiogenesis suppresses carcinoma cell invasion. Nature Med 3:1222-1227

Skobe M, Fusenig NE (1998). Tumorigenic conversion of immortal human keratinocytes through stromal cell activation. Proc Natl Acad Sci USA 95:1050-1055

Slamon D, Leyland-Jones B, Shak S, et al (1998). Addition of Herceptin (humanized anti-HER2 antibody) to first line chemotherapy for HER2 overexpressing metastatic breast cancer (HER+/MBC) markedly increases anticancer activity: A randomised, multinational controlled phase III trial. Proc Am Soc Clin Oncol 17:98a (abstract 377)

Smith MR, Kung H, Durum SK, et al (1997). TIMP-3 induces cell death by stabilizing TNF-α receptors on the surface of human colon carcinoma cells. Cytokine 9:770-780

Smythe WR, Wasfi D, Bavaria JE, et al (1997). Loss of alpha-v integrin expression and recurrence in node-negative lung carcinoma. Ann Thorac Surg 64:949-953

Soini Y, Paakko P, Autio-Harmainen H (1993). Genes of laminin B1 chain, α1 (IV) chain of type IV collagen, and 72kd type IV collagenase are mainly expressed by the stromal cells of lung carcinomas. Am J Pathol 142:1622-1630

Sotos GA, Grogan L, Allegra CJ (1994). Preclinical and clinical aspects of biomodulation of 5-fluorouracil. Cancer Treatment Rev 20:11-49

Sozzi G, Miozzo M, Denghi R, et al (1992). Deletions of 17p and p53 mutations in preneoplastic lesions of the lung. Cancer Res 52:6079-6082

Stavri GT, Zachary IC, Baskerville PA, et al (1995). Basic fibroblast growth factor upregulates the expression of vascular endothelial growth factor in vascular smooth muscle cells. Synergistic interaction with hypoxia. Circulation 92:11-14

Stetler-Stevenson WG, Krutsch FIC, Liotta LA (1989). Tissue inhibitor of metalloproteinase (TIMP-2). J Biol Chem 264:17374-17378

Stetler-Stevenson WG, Hewitt R, Corcoran M (1996). Matrix metalloproteinases and tumour invasion: from correlation and causality to the clinic. Cancer Biol 7:147-154

Stricklin GP, Welgus HG (1983). Human skin fibroblast collagenase inhibitor. Purification and biochemical characterization. J Biol Chem 258:12252-12258

Stromblad S, Becker JC, Yebra M, et al (1996). Suppression of p53 and $p21^{WAF1/CIP1}$ expression by vascular cell integrin $\alpha v\beta_3$ during angiogenesis. J Cell Sci 98:426-433

Strongin AY, Collier I, Bannikov BL, et al (1995). Mechanism of cell surface activation of 72 kDa type IV collagenase. J Biol Chem 270:5331-5338

Sulzer MA, Leers MP, van Noord JA, et al (1998). Reduced E-cadherin expression is associated with increased lymph-node metastasis and unfavorable prognosis in non-small cell lung cancer. Am J Respir Crit Care Med 157:1319-1323

Suzuki M, Iizasa T, Fujisawa T, et al (1999). Expression of matrix metalloproteinases and tissue inhibitor of matrix metalloproteinases in non-small-cell lung cancer. Invasion Metastasis 18:134-141

Suzuma L, Takagi H, Otani A, et al (1998). Hypoxia and vascular endothelial growth factor stimulate angiogenic integrin expression in bovine retinal microvascular endothelial cells. Invest Ophthalmol Vis Sci 39:1035-1038

Takahashi Y, Kitadai Y, Bucana CD, et al (1995). Expression of vascular endothelial growth factor and its receptor, KDR, correlates with vascularity, metastases and proliferation of human colon cancer. Cancer Res 55:3964-3968

Takahashi Y, Kawahara F, Noguchi M, et al (1999). Activation of matrix metalloproteinase-2 in human breast cancer cells overexpressing cyclooxygenase-1 or -2. FEBS Lett 460:145-148

Takanami I, Imamura T, Hashizume K, et al (1996). Immunohistochemical detection of basic fibroblast growth factor as a prognostic indicator in pulmonary adenocarcinoma. Jpn J Clin Oncol 26:293-297

Takanami I, Tanaka F, Hashizume T, et al (1997). Pulmonary adenocarcinoma angiogenesis: a study of factors that may influence tumor angiogenesis and survival. Int J Oncol 10:101-106

Takigawa M, Nishida Y, Suzuki F, et al (1990). Induction of angiogenesis in chick yolk-sac membrane by polyamines and its inhibition by tissue inhibitors of metalloproteinases (TIMP-1 and TIMP-2). Biochim Biophys Res Commun 171:1264-1271

Talbot DC, Brown PD (1996). Experimental and clinical studies on the use of matrix metalloproteinase inhibitors for the treatment of cancer. Eur J Cancer 32A:2528-2533

Tamargo RJ, Bok RA, Brem H (1991). Angiogenesis inhibition by minocycline. Cancer Res 51:672-675

Tanaka H, Nishida K, Sugita K, et al (1999). Antitumor efficacy of hypothemycin, a new Ras-signaling inhibitor. Jpn J Cancer Res 90:1139-45

Tanigawa N, Amaya H, Matsumura M, et al (1996). Extent of tumour vascularization correlates with prognosis and hematogenous metastasis in gastric carcinomas. Cancer Res 56:2671-2676

Taraboletti G, Gardfalo A, Belotti O, et al (1995). Inhibition of angiogenesis and murine angioma growth by batimastat, a synthetic inhibitor of matrix metalloproteinases. J Natl Cancer Inst 87:293-298

Tateishi M, Ishida T, Mitsudomi T, et al, (1991). Prognostic value of c-erbB-2 protein expression in human lung adenocarcinoma and squamous cell carcinoma. Eur J Cancer 27:1372-1375

Teicher BA, Holden SA, Dupuis NA, et al (1994). Potentiation of cytotoxic therapies by TNP-470 alone and with other anti-angiogenic agents. Int J Cancer 57:920-925

Terman BI, Dougher VM, Carrion ME, et al (1992). Identification of the KDR tyrosine kinase as a receptor for vascular endothelial cell growth factor. Biochem Biophys Res Commun 187:1579-1586

Testa JE (1992). Loss of the metastatic phenotype by a human epidermoid carcinoma cell line, HEp-3, is accompanied by increased expression of tissue inhibitor of metalloproteinase 2. Cancer Res 52:5597-5603

Thomas P, Khokha R, Shepherd FA, et al (2000). Differential expression of matrix metalloproteinases and their inhibitors in non-small cell lung cancer. J Pathol 190:150-156.

Tischer E, Mitchell R, Hartman T, et al (1991). The human gene for vascular endothelial growth factor. Multiple protein forms are encoded through alternative exon splicing. J Biol Chem 266:11947-11954

Toi M, Kashitani J, Tominaga T (1993). Tumour angiogenesis is an independent prognostic indicator in primary breast carcinoma. Int J Cancer 55:371-374

Toi M, Inada K, Hoshina S, et al (1995a). Vascular endothelial growth factor and platelet-derived endothelial cell growth factor are frequently coexpressed in highly vascularized human breast cancer. Clin Cancer Res 36:193-204

Toi M, Hoshima S, Taniguchi T, et al (1995b). Expression of platelet-derived endothelial cell growth factor/thymidine phosphorylase in human breast cancer. Int J Cancer 64:79-82

Tokaraku M, Sato H, Murakami S, et al (1995). Activation of the precursors of gelatinase A/72kDa type IV collagenase/MMP-2 in lung carcinomas correlates with the expression of membrane-type matrix metalloproteinases (MT-MMP) and with lymph node metastasis. Int J Cancer 64:355-359

Tolnay E, Wiethege T, Kuhnen C, et al (1997). Expression of type IV collagenase correlates with the expression of vascular endothelial growth factor in primary non-small cell lung cancer. J Cancer Res Clin Oncol 123:652-658

Tomita T, Iwata K (1996). Matrix metalloproteinases and tissue inhibitors of metalloproteinases in colonic adenomas-adenocarcinomas. Dis Colon Rectum 39:1255-1264

Tomozawa S, Nagawa H, Tsuno N, et al (1999). Inhibition of haematogenous metastasis of colon cancer in mice by a selective COX-2 inhibitor, JTE-522. Br J Cancer 81:1274-1279

Top B, Mooi WJ, Klaver SG, et al (1995). Comparative analysis of p53 gene mutations and protein accumulation in human non-small cell lung cancer. Int J Cancer 64:83-91

Towle MJ, Lee A, Maduakor EC, et al (1993). Inhibition of urokinase by 4-substituted benzo[b] thiophene-2 carboxamidines: an important new class of selective synthetic urokinase inhibitor. Cancer Res 53:2553-2559

Toyoyama H, Nuruki K, Ogawa H, et al (1999). The reduced expression of e-cadherin, alpha-catenin and gamma-catenin but not beta-catenin in human lung cancer. Oncology Reports 6:81-85

Tsai J-C, Goldman CK, Gillespie GY (1995). Vascular endothelial growth factor in human glioma cell lines: induced secretion by EGF, PDGF-BB, and bFGF. J Neurosurg 82:864-873

Tsuji M, DuBois RN (1995). Alterations in cellular adhesion and apoptosis in epithelial cells overexpressing prostaglandin endoperoxide synthase 2. Cell 83:493-501

Tsuji M, Kawano S, DuBois RN (1997). Cyclooxygenase-2 expression in human colon cancer cells increases metastatic potential. Proc Natl Acad Sci USA 94:3330-3340.

Tsuji M, Kawano S, Tsuji S, et al (1998). Cyclooxygenase regulates angiogenesis induced by colon cancer cells. Cell 93:705-716

Unemori EN, Ferrara N, Bauer EA, et al (1992). Vascular endothelial cell growth factor induces interstitial collagenase expression in human endothelial cells. J Cell Physiol 153:557-562

Unemori EN, Ehsani N, Wang M, et al (1994). Interleukin-1 and transforming growth factor-alpha: synergistic stimulation of metalloproteinases, PGE2, and proliferation in human fibroblasts. Exp Cell Res 210:166-171

Urbanski SJ, Edwards DR, Maitland A, et al (1992). Expression of metalloproteinases and their inhibitors in primary pulmonary carcinomas. Br J Cancer 66:1188-1194

Valente P, Fassina G, Melchiori A, et al (1998). TIMP-2 overexpression reduces invasion and angiogenesis and protects B16F10 melanoma cells from apoptosis. Int J Cancer 75:246-253

Van Wart H, Birkedal-Hansen H (1990). The cysteine switch: a principal regulation of metalloproteinase activity with potential applicability to the entire matrix metalloproteinase gene family. Proc Natl Acad Sci USA 87:5578-5582

van Zandwijk N, Giaccone G (1996). Treatment of metastatic non-small cell lung cancer. Curr Opin Oncol 8:120-125

Vaupel P (1997). The influence of tumor blood flow and microenvironmental factors on the efficacy of radiation, drugs and localized hyperthermia. Klin Padiatr 209:243-249

Veale D, Kerr N, Gibson GJ, et al (1993). The relationship of quantitative epidermal growth factor receptor expression in non-small cell lung cancer to long term survival. Br J Cancer 68:162-165

Verheul HMW, Hoekman K, Luykx-de Bakker S, et al (1997). Platelet: transporter of vascular endothelial growth factor. Clin Cancer Res 3:2187-2190

Vermeulen PB, Gasparini G, Fox SB, et al (1996). Quantification of angiogenesis in solid human tumours: an international consensus on the methodology and criteria of evaluation. Eur J Cancer 32A:2474-2484

Vermeulen PB, Libura M, Libura J, et al (1997). Influence of investigator experience and microscopic field size on microvessel density in node-negative breast carcinoma. Breast Cancer Res Treat 42:165-172

Vincent PW, Bridges AJ, Dykes DJ, et al (2000). Anticancer efficacy of the irreversible EGFr tyrosine kinase inhibitor PD 0169414 against human tumor xenografts. Cancer Chemother Pharmacol 45:231-238

Vlodavsky I, Bar-Shavit R, Ishai-Michaeli R, et al (1991a). Extracellular sequestration and release of fibroblast growth factor: a regulatory mechanism? Trends Biochem Sci 16:268-271

Vlodavsky I, Fuks Z, Ashai-Michaeli R, et al (1991b). Extracellular matrix-resident basic fibroblast growth factor: implications for control of angiogenesis. J Cell Biochem 47:167-176

Vokes EE (1997). CHART for non-small-cell lung cancer - promises and limitations. Lancet 350:156-157

Volm M, Drings P, Wodrich W (1993). Prognostic significance of the expression of c-fos, c-jun and c-erbB-1 oncogene products in human squamous cell lung carcinomas. J Cancer Res Clin Oncol 119:507-510

Volm M, Koomagi R, Mattern J, et al (1997). Prognostic value of basic fibroblast growth factor and its receptor (FGFR-1) in patients with non-small cell lung carcinomas. Eur J Cancer 33:691-693

Walker C, Robertson L, Myskow M, et al (1995). Expression of the Bcl-2 protein in normal and dysplastic bronchial epithelium and in lung carcinomas. Br J Cancer 72:164-169

Walker C, Robertson L, Myskow M, et al (1995). Expression of the Bcl-2 protein in normal and dysplastic bronchial epithelium and in lung carcinomas. Br J Cancer 72:164-169

Walker RA (1998). The erbB/HER type 1 tyrosine kinase receptor family. J Pathol 185:234-235

Waltenberger J, Mayr U, Pentz S, et al (1996). Functional upregulation of vascular endothelial growth factor receptor KDR by hypoxia. Circulation 94:1647-1654

Wang M, Liu YE, Greene J, et al (1997). Inhibition of tumour growth and metastasis of human breast cancer cells transfected with tissue inhibitor of metalloproteinase 4. Oncogene 14:2767-2774

Wang G, Dong Z, Xu G, et al (1998). The effect of antibody against vascular endothelial growth factor on tumour growth and metastasis. J Cancer Res Clin Oncol 124:615-620

Weich H, Iberg N, Klagsbrun M, et al (1991). Transcriptional regulation of fibroblast growth factor gene expression in capillary endothelial cells. J Cell Biochem 47:158-194

Weidner N, Semple JP, Welch WR, et al (1991). Tumour angiogenesis and metastasis- correlation in invasive breast carcinoma. N Engl J Med 324:1-8

Weidner N, Folkman J, Pozza F, et al (1992). Tumour angiogenesis: a new significant and independent prognostic indicator in early stage breast carcinoma. J Natl Cancer Inst 84:1875-1887

Weidner N, Carroll PR, Flax J, et al (1993). Tumour angiogenesis correlates with metastasis in invasive prostate carcinoma. Am J Pathol 143:401-409

Weiner DB, Nordberg J, Robinson R, et al (1990). Expression of the neu gene-encoded protein (p185[neu]) in human non-small cell carcinomas of the lung. Cancer Res 50:421-5

Weinstat-Saslow DL, Zabrenetsky VS, Van HK, et al (1994). Transfection of thrombospondin 1 complementary DNA into human breast carcinoma line reduces primary growth, metastatic potential and angiogenesis. Cancer Res 54:6504-6511

Wells A, Gupta K, Chang P, et al (1998). Epidermal growth factor receptor-mediated motility in fibroblasts. Microsc Res Tech 43:395-411

Wenger RH, Gassmann M (1997). Oxygen(s) and the hypoxia-inducible factor-1. Biol Chem 378:609-616

Wernert N, Raes MB, Lassalle P, et al (1992). c-ets1 proto-oncogene is a transcrition factor expressed in endothelial cells during tumor vascularization and other forms of angiogenesis in humans. Am J Pathol 140:119-127

Wernert N, Gilles F, Fafeur V, et al (1994). Stromal expression of c-Ets1 transcription factor correlates with tumour invasion. Cancer Res 54:5683-5688

Westermarck J, Kahari V-M (1999). Regulation of matrix metsalloproteinase expression in tumor invasion. FASEB J 13;781-792

Wick W, Wagner S, Kerkau S, et al (1998). BCL-2 promotes migration and invasiveness of human gliomal cells. FEBS Lett 440:419-424

Wilkins HE 3rd, Connolly MM, Grays P, et al (1997). Recombination interferon alpha-2b in the management of malignant pleural effusions. Chest 111:1597-1599

Wilson CL, Heppner KJ, Labosky PA, et al (1997). Intestinal tumorigenesis is suppressed in mice lacking the metalloproteinase matrilysin. Proc Nat Acad Sci USA 94:1402-1407

Wingo PA, Ries LAG, Giovino GA, et al (1999). Annual report to the nation on the status of cancer, 1973-1996, with a special section on lung cancer and tobacco smoking. J Nat Cancer Inst 8:675-690

Witty JP, McDonnell S, Newell K, et al (1994). Modulation of matrilysin levels in colon carcinoma cell lines affect tumorigenicity in vivo. Cancer Res 54:4805-4812

Wojtowicz-Praga SM, Dickson RM, Hawkins MJ (1997). Matrix metalloproteinase inhibitors. Invest NewDrugs 15:61-75

Wolf DC, Gross EA, Lyght O, et al (1995). Immunohistochemical localization of p53,PCNA and TGF-α protein in formaldehyde-induced rat nasal squamous cell carcinomas. Toxicol Appl Pharmacol 132:27-35

Wolff H, Saukkonen K, Anttila S, et al (1998). Expression of cyclooxygenase-2 in human lung carcinoma. Cancer Res 15:4997-5001

Wright C, Angus B, Nicolson S, et al (1989). Expression of the c-erbB-2 oncoprotein: a prognostic indicator in human breast cancer. Cancer Res 49:2087-90

Yamasaki K, Abe S, Takekawa H, et al (1994). Tumour angiogenesis in human lung adenocarcinoma. Cancer 74:2245-2250

Yan SF, Tritto I, Pinsky D, et al (1995). Induction of interleukin 6 (IL-6) by hypoxia in vascular cells. J Biol Chem 270:11463-11471

Yanase T, Tamura M, Fujita K, et al (1993). Inhibitory effect of angiogenesis inhibitor TNP-470 on tumor growth and metastasis of human cell lines in vitro and in vivo. Cancer Res 53:2566-2570

Yano T, Tanikawa S, Fujie T, et al (2000). Vascular endothelial growth factor expression and neovascularisation in non-small cell lung cancer. Eur J Cancer 36:601-609

Ylisirnio S, Hoyhtya M, Turpeenniemi-Hujanen T, et al (2000). Serum matrix metalloproteinases −2, −9 and tissue inhibitors of metalloproteinases −1, −2 in lung cancer -TIMP-1 as a prognostic marker. Anticancer Res 20;1311-1316

Yoshimura A, Kawazuru Y, Furukawa T, et al (1990). Purification and tissue distribution of human thymidine phosphorylase; high expression in lymphocytes, reticulocytes and tumors. Biochim Biophys Acta 1034:107-113

Yuan A, Yang PC, Yu CJ, et al (1995). Tumour angiogenesis corelates with histological type and metastasis in non-small-cell lung cancer. Am J Respir Crit Care Med 152:2157-2162

Yucel-Lindberg T, Ahola H, Carlstedt-Duke J, et al (1999). Involvement of tyrosine kinases on cyclooxygenase expression and prostaglandin E2 production in human gingival fibroblasts stimulated with interleukin-1beta and epidermal growth factor. Biochem Biophys Res Commun 257:528-532

Yurchenco PD, Cheng YS, Colognato H (1992). Laminin forms an independent network in basement membranes. J Cell Biol 117:1119-1133

Zabrenetzky V, Harris CC, Steeg PS, et al (1994). Expression of the extracellular matrix molecule thrombospondin inversely correlates with malignant progression in melanoma, lung and breast carcinoma cell lines. Int J Cancer 59:191-195

Zeng ZS, Cohen AM, Zhang ZF, et al (1995). Elevated tissue inhibitor of metalloproteinase-1 (TIMP-1) RNA in colorectal cancer stroma correlates with lymph node and distant metastasis. Clin Cancer Res 1:899-906

Zhang Y, Deng Y, Luther T, et al (1994). Tissue factor controls the balance of angiogenic and anti-angiogenic properties of tumor cells in mice. J Clin Invest 9:1320-1327

Zhang HT, Craft P, Scott PA, et al (1995). Enhancement of tumour growth and vascular density by transfection of vascular endothelial cell growth factor into MCF-7 human breast carcinoma cells. J Natl Cancer Inst 87:213-219

Zucker S, Lysic RM, Malik M, et al (1992). Secretion of gelatinases and tissue inhibitors of metalloproteinases by human lung cancer cell-lines and revertant cell-lines: not an invariant correlation with metastasis. Int J Cancer 52:366-371

Zucker S, Mirza H, Conner CE, et al (1998). Vascular endothelial growth factor induces tissue factor and matrix metalloproteinase production in endothelial cells: conversion of prothrombin to thrombin results in progelatinase A activation and cell proliferation. Int J Cancer 75:780-786

List of Publications Relating to Thesis

Original papers

<u>Cox G</u>, Jones JL, O'Byrne KJ (2000). MMP-9 and the epidermal growth factor receptor signal pathway in operable non-small cell lung cancer. Clin Cancer Res 6:2349-2355

<u>Cox G</u>, Walker RA, Andi A, Steward WP, O'Byrne KJ. Prognostic significance of platelet and microvessel counts in operable non-small cell lung cancer. Lung Cancer; *in press*

<u>Cox G</u>, Walker RA, Muller S, Abrams KR, Steward WP, O'Byrne KJ. Does immunointensity account for the differences in prognostic significance of Bcl-2 expression in non-small cell lung cancer? Pathol Oncol Res; *in press*

<u>Cox G</u>, Vyberg M, Melgaard B, Askaa J, Oster A, O'Byrne KJ. HercepTest™ HER2 expression and gene amplification in non-small cell lung cancer. Int J Cancer; *in press*

Review articles

<u>Cox G</u>, Steward WP, O'Byrne KJ (1999). The plasmin cascade and matrix metalloproteinases in non-small cell lung cancer. Thorax 54:169-179

<u>Cox G</u>, Jones JL, Walker RA, Steward WP, O'Byrne KJ (2000). Angiogenesis and non-small cell lung cancer. Lung Cancer 27:81-100

Submitted manuscript

<u>Cox G</u>, Jones JL, Andi A and O'Byrne KJ Molecular biological staging system for operable non-small cell lung cancer. Thorax

Invited review

<u>Cox G</u>, O'Byrne KJ. Matrix metalloproteinases in cancer. Anticancer Res

Associated papers

Koukourakis MI, Giatromanolaki A, O'Byrne KJ, <u>Cox G</u>, Krammer B, Gatter KC, Harris AL (1999). bcl-2 and c-erbB-2 proteins are involved in the regulation of VEGF and of thymidine phosphorylase angiogenic activity in non-small cell lung cancer. Clin Exp Metastasis 17:545-554

O'Byrne KJ, Koukourakis MI, Giatromanolaki A, <u>Cox G</u>, Thurley H, Steward WP, Gatter K, Harris AL (2000).Vascular endothelial growth factor, platelet-derived endothelial cell growth factor and angiogenesis in non-small cell lung cancer. Br J Cancer 82:1427-1432

Giatromanolaki A, Koukourakis MI, Sivridis E, O'Byrne K, <u>Cox G</u>, Thorpe PE, Gatter KC, Harris AL (2000). Coexpression of MUC1 glycoprotein with multiple angiogenic factors in non-small cell lung cancer suggests coactivation of angiogenic and migration pathways. Clin Cancer Res 6:1917-1920

Published Abstracts

Cox G, Walker RA, O'Byrne KJ (1999). Bcl-2 immunointensity confers differing prognosis in non-small cell lung cancer. Am J Resp Med 159(3):A202

Cox G, Walker RA, Steward WP, O'Byrne KJ (1999). Prognostic significance of platelet and microvessel counts in non-small cell lung cancer. Br J Cancer 80SII:89

Cox G, Walker RA, O'Byrne KJ (1999). Increased platelet and microvessel counts confer poor prognosis in operable non-small cell lung cancer. Thorax 54S:A20

Cox G, Jones JL, O'Byrne KJ (1999). MMP-9 and the epidermal growth factor signal pathway in non-small cell lung cancer. Thorax 54S:A82

Cox G, Jones JL, O'Byrne KJ (1999). Expression and prognostic influence of MMP-2, MMP-9 and TIMP-2 in non-small cell lung cancer. Thorax 54S:A83

Cox G, Jones JL, Andi A, O'Byrne KJ (2000). Molecular staging for operable non-small cell lung cancer. J Clin Oncol 19:482a

Cox G, Vyberg M, Melgaard B, O'Byrne KJ (2000). HER2 gene amplification and protein expression in non-small cell lung cancer. Thorax 55S