Angiogenesis and Matrix Metalloproteinases
in Non-Small Cell Lung Cancer

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by

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Abstract

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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

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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
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.

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<td>platelet activating factor (PAF)</td>
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<td>PGE1, PGE2</td>
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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.

- ECM degradation
- Migration of endothelial cells towards angiogenic stimulus
- Tubule formation
- Degradation of the ECM by proteases released from tumour and activated endothelial cells
- Angiogenic stimulators
- Formation of capillary bud
- Proliferation leading to
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.**


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,
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.

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
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 chemotaetic 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 cytoskeleton-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 (Ocharoenrat 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
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 G1-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 positive 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-expression (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 G1 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).


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

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 phosphorylation 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 αvβ3 is closely linked with angiogenesis. It is expressed in growing vessels and is upregulated in response to pro-angiogenic molecules such as TNFα, bFGF (Brooks PC et al, 1994) and VEGF (Suzuma L et al, 1998). αvβ3 binds gelatinase A (MMP-2) and will localize proteolysis to the cell edge (Brooks PC et al, 1996). Antibodies to αvβ3 decrease blood vessel growth in chick chorioallantoic membrane assays (Brooks PC et al, 1994). The expression of αvβ3 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 αv-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 β1 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 β1 chain and induce signal transduction. The β1 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 β1 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 PGE2 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.

<table>
<thead>
<tr>
<th>Major subsets</th>
<th>Name</th>
<th>MMP no.</th>
<th>major substrates</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CLASSIC MATRIX METALLOPROTEINASE (MMP) MEMBERS:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>COLLAGENASES</td>
<td>interstitial collagenase</td>
<td>MMP-1</td>
<td>fibrillar collagens</td>
</tr>
<tr>
<td></td>
<td>neutrophil collagenase</td>
<td>MMP-8</td>
<td>fibrillar collagens</td>
</tr>
<tr>
<td></td>
<td>collagenase-3</td>
<td>MMP-13</td>
<td>fibrillar collagens</td>
</tr>
<tr>
<td></td>
<td>?</td>
<td>MMP-18</td>
<td>collagens I, II, III, VII</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>denatured collagen</td>
</tr>
<tr>
<td>STROMELYINS</td>
<td>stromelysin-1</td>
<td>MMP-3</td>
<td>laminin, fibronectin, proteoglycans, type IV collagen</td>
</tr>
<tr>
<td></td>
<td>stromelysin-2</td>
<td>MMP-10</td>
<td>type IV collagen</td>
</tr>
<tr>
<td></td>
<td>matrilysin (PUMP-1)</td>
<td>MMP-7</td>
<td>type IV collagen, proteoglycans, type IV collagen, gelatins, ECM glycoproteins, elastin</td>
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<tr>
<td>GELATINASES</td>
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<td>type I &amp; IV collagen, gelatin</td>
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<td></td>
<td>gelatinase B</td>
<td>MMP-9</td>
<td>type IV &amp; V collagen, gelatin</td>
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<td>MMP-12</td>
<td>elastin</td>
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<td>?</td>
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<tr>
<td>RXKR SECRETED TYPE</td>
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<td>RXKR MEMBRANE TYPE</td>
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<td>proMMP-2, laminin</td>
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<td></td>
<td>MT5-MMP</td>
<td>MMP-21</td>
<td>proMMP-2</td>
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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.

<table>
<thead>
<tr>
<th>TIMP</th>
<th>properties</th>
<th>preferred binding site</th>
<th>other binding sites</th>
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</thead>
<tbody>
<tr>
<td>TIMP-1</td>
<td>29kDa</td>
<td>proMMP-9, MMP-9</td>
<td>MMP-1, MMP-2, MMP-3</td>
</tr>
<tr>
<td>TIMP-2</td>
<td>21kDa</td>
<td>proMMP-2, MMP-2, MT1-MMP</td>
<td>MMP-1, MMP-3, MMP-9</td>
</tr>
<tr>
<td>TIMP-3</td>
<td>27kDa</td>
<td>MMP-1, MMP-3</td>
<td>MMP-2, MMP-9</td>
</tr>
<tr>
<td>TIMP-4</td>
<td>23kDa</td>
<td>MMP-2</td>
<td>MMP-1, MMP-3, MMP-7, MMP-9</td>
</tr>
</tbody>
</table>

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), NFkB (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
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 αvβ3 is capable of binding MMP-2 (Brooks PC et al, 1996). αvβ3 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.
MMP-9 expression is strongly associated with the metastatic ability of rat embryo fibroblasts; overexpression 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
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 associated 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 αvβ3 (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 receptor 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.
MMPs in non-small cell lung cancer

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 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 immunoeexpression 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,

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 (Müller 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 $\alpha_1$ 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 immunohistoexpressed 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 immunohistoexpression 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)(Rec 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


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 cavity but 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.
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 IgG1) 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 IgG2a) 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).


*MMP-9 (Gelatinase B)*: anti-MMP-9 mouse monoclonal antibody (Chemicon International Ltd). Clone 56-2A4 (isotype IgG1k) recognises residues 626-644 of the carboxy terminal domain of both latent and active MMP-9 (Nomura H et al, 1996).


*TIMP-1*: anti-TIMP-1 mouse monoclonal antibody (Chemicon International Ltd). Clone 147-6D11 (isotype IgG1k) specifically reacts with human TIMP-1 (Fukuda Y et al, 1991).

*TIMP-2*: anti-TIMP-2 mouse monoclonal antibody (Chemicon International Ltd). Clone 67-6H11 (isotype IgG1k) 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)
25cm² and 75cm² 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 (CH₃COCH₃) (Fisher Scientific)
3-aminopropytriethoxysilane 99% (C₉H₂₃NO₃Si) (Acros Organics)
anhydrous di-sodium hydrogen orthophosphate (Na₂HPO₄·H₂O) (Fisons)
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
130°C 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. Streptavidin 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 75cm² 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:

<table>
<thead>
<tr>
<th>TBS:</th>
<th>1000μl</th>
</tr>
</thead>
<tbody>
<tr>
<td>streptavidin:</td>
<td>1μl</td>
</tr>
<tr>
<td>biotinylated horseradish peroxidase:</td>
<td>1μl</td>
</tr>
</tbody>
</table>

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

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

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.

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

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)
腺癌
RPMI + 10% FBS
分裂1:10每周两次

**A549** (Chinje EC et al, 1999)
腺癌
DMEM + 10% FBS
分裂1:10每周

**MOR-P** (Rhodes T et al, 1992)
腺癌
RPMI + 10% FBS
分裂1:10每周

**SK-MES** (Favoni RE et al, 1994)
鳞状细胞癌
DMEM + 10% FBS
分裂1:10每周
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% CO₂/95% 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 25cm² and 75cm² 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 25cm², 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 10⁴ cells per cm².

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 25cm² flask and placed in a 1.8ml cryotube.

**Stimulation with EGF.**
2x10⁵ 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)\textsubscript{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 cofactors 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\textsuperscript{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)\textsubscript{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)\textsubscript{25} Dynabeads and mixed thoroughly with gentle pipetting. To allow annealing between oligo (dT)\textsubscript{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 10⁶ cells is between 600 and 1600μg. mRNA represents 1-5% of total RNA therefore the mRNA yield from 10⁵ 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.

<table>
<thead>
<tr>
<th></th>
<th>RT +ve</th>
<th>RT -ve</th>
</tr>
</thead>
<tbody>
<tr>
<td>5x AMV RT buffer*</td>
<td>55μl</td>
<td>55μl</td>
</tr>
<tr>
<td>dNTP mix (10mM) *</td>
<td>44μl</td>
<td>44μl</td>
</tr>
<tr>
<td>Rnasln (40U/μl)*</td>
<td>11μl</td>
<td>11μl</td>
</tr>
<tr>
<td>AMV RT (10U/μl)*</td>
<td>8.8μl</td>
<td>-</td>
</tr>
<tr>
<td>PCR grade H₂O</td>
<td>46.2μl</td>
<td>55μl</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td>165μl</td>
<td>165μl</td>
</tr>
</tbody>
</table>
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:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x AJ buffer*</td>
<td>50μl</td>
</tr>
<tr>
<td>forward &amp; reverse primers (10pmole/μl)</td>
<td>10μl each</td>
</tr>
<tr>
<td>PCR grade H₂O for GAPDH</td>
<td>420μl</td>
</tr>
<tr>
<td>PCR grade H₂O for MMP-2, -9, MT1-MMP, TIMP-1 and -2</td>
<td>410μl</td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>cycle</th>
<th>denaturation</th>
<th>annealing</th>
<th>extension</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>94°C, 5 minutes</td>
<td>#°C, 1 minute</td>
<td>72°C, 1 minute</td>
</tr>
<tr>
<td>n</td>
<td>94°C, 1 minute</td>
<td>#°C, 1 minute</td>
<td>72°C, 1 minute</td>
</tr>
<tr>
<td>last</td>
<td>94°C, 1 minute</td>
<td>#°C, 1 minute</td>
<td>72°C, 7 minutes</td>
</tr>
</tbody>
</table>

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’ system. The oligonucleotides were synthesised by Gibco BRL, Oswick and Genosys, and the forward primer of each primer set was synthesised biotinylated.

Table 2.3 Oligonucleotide primer sequences.

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

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
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. 5x10⁴ 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 0.25mm², 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 Na₃H₂PO₄, 140mM NaH₂PO₄

*citric acid buffer*

10mM citric acid, pH 6.0

*DAB solution*

9ml de-ionised water
500μl diaminobenzidine tetrahydrochloride solution
500μl 20xTBS
100μl 3% H₂O₂

*Mayers Haematoxylin*

105mM aluminium potassium sulphate (AlK(SO₄)₂.12H₂O)
5mM citric acid (C₆H₅Cl₂O₇)
303mM chloral hydrate (C₆H₅Cl₂O₂)
1mM sodium iodate (NaIO₃)

*Eosin solution*

1% aqueous water soluble eosin

**Epithelial cell culture.**

*freezing media (10ml)*

5ml FBS
0.5ml 200nM L-glutamine
0.75ml dimethyl sulfoxide (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% NaN₃. In accordance with the manufacturers instructions 30μl beads (150μg) were used to extract mRNA from 10⁵ 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)**

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

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

**Washing Buffer with SDS (200ml)**

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

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

**Washing Buffer (200ml)**

<table>
<thead>
<tr>
<th>Stock</th>
<th>Concentration</th>
<th>Vol. Stock</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCl pH 8.0</td>
<td>1M</td>
<td>2ml</td>
<td>0.01M</td>
</tr>
<tr>
<td>LiCl</td>
<td>1.5M</td>
<td>20ml</td>
<td>0.15M</td>
</tr>
<tr>
<td>EDTA</td>
<td>0.5M</td>
<td>40µl</td>
<td>0.001M</td>
</tr>
</tbody>
</table>
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 MgCl₂
- 50mM dithiothreitol (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*

<table>
<thead>
<tr>
<th>Component</th>
<th>Stock Concentration</th>
<th>Vol. Stock</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCl pH 8.0</td>
<td>1M</td>
<td>2250µl</td>
<td>45mM</td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td>1M</td>
<td>550µl</td>
<td>11mM</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>1M</td>
<td>225µl</td>
<td>4.5mM</td>
</tr>
<tr>
<td>each dNTP</td>
<td>100mM</td>
<td>100µl</td>
<td>200µM</td>
</tr>
<tr>
<td>bovine serum albumin</td>
<td>20mg/ml</td>
<td>275µl</td>
<td>110µg/ml</td>
</tr>
<tr>
<td>β Mercaptoethanol</td>
<td>14.3M</td>
<td>23.4µl</td>
<td>6.7mM</td>
</tr>
<tr>
<td>EDTA pH 8.0</td>
<td>0.01M</td>
<td>2.2µl</td>
<td>4.4µM</td>
</tr>
</tbody>
</table>

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 eyepiece 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 (≥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 344x10^9/l, SD 122 x10^9/l, median 320 x10^9/l, range 92 to 910 x10^9/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 x10^9/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).

<table>
<thead>
<tr>
<th>prognostic factor</th>
<th>no.</th>
<th>%</th>
<th>log-rank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age- (years)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mean-</td>
<td>64.5</td>
<td>(SD 7.57)</td>
<td></td>
</tr>
<tr>
<td>median-</td>
<td>66 years</td>
<td></td>
<td>p=0.62</td>
</tr>
<tr>
<td>range-</td>
<td>42-78 years</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>male-</td>
<td>128</td>
<td>70.7%</td>
<td>p=0.19</td>
</tr>
<tr>
<td>female-</td>
<td>53</td>
<td>29.3%</td>
<td></td>
</tr>
<tr>
<td>Histology-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>squamous-</td>
<td>113</td>
<td>62.4%</td>
<td>p=0.96</td>
</tr>
<tr>
<td>adenocarcinoma-</td>
<td>54</td>
<td>29.8%</td>
<td></td>
</tr>
<tr>
<td>large cell-</td>
<td>14</td>
<td>7.7%</td>
<td></td>
</tr>
<tr>
<td>Grade-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>well/moderate-</td>
<td>92</td>
<td>50.8%</td>
<td>p=0.36</td>
</tr>
<tr>
<td>poorly differentiated-</td>
<td>89</td>
<td>49.2%</td>
<td></td>
</tr>
<tr>
<td>Stage-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I-</td>
<td>88</td>
<td>48.6%</td>
<td>p=0.0001</td>
</tr>
<tr>
<td>II-</td>
<td>51</td>
<td>28.2%</td>
<td></td>
</tr>
<tr>
<td>IIIa-</td>
<td>42</td>
<td>23.2%</td>
<td></td>
</tr>
<tr>
<td>T-</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>1-</td>
<td>33</td>
<td>18.2%</td>
<td>p=0.22</td>
</tr>
<tr>
<td>2-</td>
<td>129</td>
<td>71.3%</td>
<td></td>
</tr>
<tr>
<td>3-</td>
<td>19</td>
<td>10.5%</td>
<td></td>
</tr>
<tr>
<td>N-</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>0-</td>
<td>93</td>
<td>51.4%</td>
<td>p&lt;0.0001</td>
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<tr>
<td>1-</td>
<td>56</td>
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<td></td>
</tr>
<tr>
<td>2-</td>
<td>32</td>
<td>17.7%</td>
<td></td>
</tr>
<tr>
<td>Platelet count- (n=175)(x10^9/l)</td>
<td>mean 344 (SD 122)</td>
<td>median 320 (range 92-910)</td>
<td></td>
</tr>
<tr>
<td>&gt;median-</td>
<td>86</td>
<td>49.1%</td>
<td>p=0.01</td>
</tr>
<tr>
<td>≤median-</td>
<td>89</td>
<td>50.9%</td>
<td></td>
</tr>
<tr>
<td>Chalkley count- (n=180)</td>
<td>mean 18.2 (SD 5.29)</td>
<td>median 18 (range 8-33)</td>
<td></td>
</tr>
<tr>
<td>&gt;median-</td>
<td>86</td>
<td>47.5%</td>
<td>p=0.01</td>
</tr>
<tr>
<td>≤median-</td>
<td>95</td>
<td>52.5%</td>
<td></td>
</tr>
<tr>
<td>upper tertile (≥21)-</td>
<td>67</td>
<td>37.0%</td>
<td></td>
</tr>
<tr>
<td>lower tertiles (&lt;21)-</td>
<td>114</td>
<td>63.0%</td>
<td>p=0.0006</td>
</tr>
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</table>
Table 3.2 Relationships with platelet count (n=175).

<table>
<thead>
<tr>
<th>prognostic factor</th>
<th>platelet count</th>
<th></th>
<th>( \chi^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>below median</td>
<td>above median</td>
<td></td>
</tr>
<tr>
<td>Age-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>below median-</td>
<td>38</td>
<td>47</td>
<td>0.15</td>
</tr>
<tr>
<td>above median-</td>
<td>50</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>Sex-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>male-</td>
<td>63</td>
<td>60</td>
<td>0.70</td>
</tr>
<tr>
<td>female-</td>
<td>25</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>Histology-</td>
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<td></td>
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</tr>
<tr>
<td>squamous-</td>
<td>51</td>
<td>57</td>
<td>0.44</td>
</tr>
<tr>
<td>adenocarcinoma-</td>
<td>28</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>large cell-</td>
<td>9</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Grade-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>well/moderate-</td>
<td>40</td>
<td>49</td>
<td>0.15</td>
</tr>
<tr>
<td>poorly differentiated-</td>
<td>48</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td>Stage-</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>I-</td>
<td>48</td>
<td>37</td>
<td>0.27</td>
</tr>
<tr>
<td>II-</td>
<td>21</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>IIIa-</td>
<td>19</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>T-</td>
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<td>13</td>
<td>0.30</td>
</tr>
<tr>
<td>2-</td>
<td>61</td>
<td>63</td>
<td></td>
</tr>
<tr>
<td>3-</td>
<td>7</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>N-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-</td>
<td>50</td>
<td>40</td>
<td>0.27</td>
</tr>
<tr>
<td>1-</td>
<td>22</td>
<td>31</td>
<td></td>
</tr>
<tr>
<td>2-</td>
<td>16</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>Chalkley count- (n=174)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>below median-</td>
<td>45</td>
<td>46</td>
<td>0.76</td>
</tr>
<tr>
<td>above median-</td>
<td>43</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>lower + middle tertile-</td>
<td>53</td>
<td>57</td>
<td>0.41</td>
</tr>
<tr>
<td>upper tertile-</td>
<td>35</td>
<td>29</td>
<td></td>
</tr>
</tbody>
</table>
Figure 3.1 Immunohistochemistry for CD34 staining.

a) 'normal' lung adjacent to tumour

b) alveolar pattern

c) CD34 low microvessel 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)

**Figure 3.2b:**
Chalkley count (high vs medium/low)

**Figure 3.2c:**
Pre-Operative Platelet Count

**Figure 3.2d:**
Pre-operative Platelet Count

**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.

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

Discussion.
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\textsuperscript{PL,T}) (Salgado R et al, 1999) and increased VEGF\textsuperscript{PL,T} 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.


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

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.

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 immunexpression 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).

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

**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 N2 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
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 Clinicopathological findings for HercepTest™.

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

Pathological stage of positive cases

2+ 8 cases: Ia, Ia, Ib, Ia, IIb, IIb, IV, IV
3+ 7 cases: IIb, IIIa, IV, 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**

- **Survival (days)**

- **p = 0.29**

**Figure 4.2b:**
- **cytoplasmic p53 expression**

- **Survival (days)**

- **p = 0.26**

**Figure 4.2c:**
- **Bcl-2 expression**

- **Survival (days)**

- **p = 0.04**

**Figure 4.2d:**
- **Bcl-2 immunointensity**

- **Survival (days)**

- **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**

- **Survival (days)**

- **p = 0.35**

**Figure 4.2f:**
- **cytoplasmic EGFR expression**

- **Survival (days)**

- **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.

<table>
<thead>
<tr>
<th>HercepTest™ score</th>
<th>FISH analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>no amplification</td>
</tr>
<tr>
<td>0</td>
<td>n=7</td>
</tr>
<tr>
<td>1+</td>
<td>n=12</td>
</tr>
<tr>
<td>2+</td>
<td>n=4</td>
</tr>
<tr>
<td>3+</td>
<td></td>
</tr>
</tbody>
</table>

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).

<table>
<thead>
<tr>
<th>prognostic factor</th>
<th>Chalkley count</th>
<th>Chalkley count</th>
<th>Chalkley count</th>
<th>Chalkley count</th>
<th>$\chi^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt; median</td>
<td>&gt; median</td>
<td>lower tertile</td>
<td>upper tertile</td>
<td></td>
</tr>
<tr>
<td>nuclear p53-</td>
<td>negative</td>
<td>53</td>
<td>49</td>
<td>63</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td>positive</td>
<td>41</td>
<td>37</td>
<td>51</td>
<td>27</td>
</tr>
<tr>
<td>Bcl-2-</td>
<td>negative</td>
<td>64</td>
<td>52</td>
<td>76</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>positive</td>
<td>29</td>
<td>32</td>
<td>36</td>
<td>25</td>
</tr>
<tr>
<td>EGFR-</td>
<td>negative</td>
<td>64</td>
<td>52</td>
<td>76</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>weak</td>
<td>21</td>
<td>21</td>
<td>26</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>strong</td>
<td>8</td>
<td>11</td>
<td>10</td>
<td>9</td>
</tr>
<tr>
<td>other</td>
<td>negative</td>
<td>39</td>
<td>45</td>
<td>47</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>positive</td>
<td>55</td>
<td>41</td>
<td>67</td>
<td>29</td>
</tr>
<tr>
<td>membranous</td>
<td>negative</td>
<td>58</td>
<td>65</td>
<td>72</td>
<td>51</td>
</tr>
<tr>
<td></td>
<td>positive</td>
<td>36</td>
<td>21</td>
<td>42</td>
<td>15</td>
</tr>
</tbody>
</table>

Table 4.5 Multivariate analysis.

<table>
<thead>
<tr>
<th>factor</th>
<th>Hazard ratio</th>
<th>95% confidence interval</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>nodal status-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-</td>
<td>1.00</td>
<td></td>
<td>0.0001</td>
</tr>
<tr>
<td>1-</td>
<td>1.70</td>
<td>1.06-2.72</td>
<td></td>
</tr>
<tr>
<td>2-</td>
<td>3.11</td>
<td>1.88-5.15</td>
<td></td>
</tr>
<tr>
<td>Bcl-2 expression-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>negative-</td>
<td>1.00</td>
<td></td>
<td>0.02</td>
</tr>
<tr>
<td>weak-</td>
<td>0.49</td>
<td>0.28-0.84</td>
<td></td>
</tr>
<tr>
<td>strong-</td>
<td>1.24</td>
<td>0.64-2.37</td>
<td></td>
</tr>
<tr>
<td>grade-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>well/moderate-</td>
<td>1.00</td>
<td></td>
<td>0.15</td>
</tr>
<tr>
<td>poor-</td>
<td>1.34</td>
<td>0.90-2.00</td>
<td></td>
</tr>
</tbody>
</table>
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. HercepTest™ positive (2+ or 3+) breast tumours may respond to treatment with the novel anti-c-erbB-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
clincopathological 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.
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 reactivity 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-expressed 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**

- **Survival (days):**
  - Cumulative Survival
  - Survival (days)

MMP-2 tumour cell

- Positive
- Negative

**Survival (days):**

- Survival (days)
- p = 0.95

**Figure 5.2b:**

**MMP-2 stromal expression**

- **Survival (days):**
  - Cumulative Survival
  - Survival (days)

MMP-2 stroma

- Positive
- Negative

**Survival (days):**

- Survival (days)
- p = 0.12

**Figure 5.2c:**

**MMP-9 tumour cell expression**

- **Survival (days):**
  - Cumulative Survival
  - Survival (days)

MMP-9 tumour cell

- Positive
- Negative

**Survival (days):**

- Survival (days)
- p = 0.001

**Figure 5.2d:**

**MMP-9 stromal expression**

- **Survival (days):**
  - Cumulative Survival
  - Survival (days)

MMP-9 stroma

- Positive
- Negative

**Survival (days):**

- Survival (days)
- p = 0.99
Figure 5.2 (continued).

**figure 5.2e:** TIMP-2 tumour cell expression

![TIMP-2 tumour cell expression graph](image)

Survival (days)

Cumulative Survival

- TIMP-2 tumour cell positive
- TIMP-2 tumour cell negative

Survival (days)

\( p = 0.62 \)

**figure 5.2f:** TIMP-2 stromal expression

![TIMP-2 stromal expression graph](image)

Survival (days)

Cumulative Survival

- TIMP-2 stroma positive
- TIMP-2 stroma negative

Survival (days)

\( p = 0.68 \)

**figure 5.2g:** EGFR and MMP-9 tumour cell co-expression

![EGFR and MMP-9 tumour cell co-expression graph](image)

Survival (days)

Cumulative Survival

- EGFR/MMP-9 positive
- EGFR/MMP-9 negative

Survival (days)

\( p = 0.0001 \)

**figure 5.2h:** Membranous EGFR and MMP-9 tumour cell co-expression

![Membranous EGFR and MMP-9 tumour cell co-expression graph](image)

Survival (days)

Cumulative Survival

- memb. EGFR/MMP-9 positive
- memb. EGFR/MMP-9 negative

Survival (days)

\( p = 0.003 \)
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.

<table>
<thead>
<tr>
<th>Factor</th>
<th>HR</th>
<th>CI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>tumour cell MMP-9 expres-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>negative</td>
<td>1.00</td>
<td></td>
<td>0.003</td>
</tr>
<tr>
<td>positive</td>
<td>1.94</td>
<td>1.25-3.04</td>
<td></td>
</tr>
<tr>
<td>Bcl-2 expression-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>negative</td>
<td>1.00</td>
<td></td>
<td>0.01</td>
</tr>
<tr>
<td>positive</td>
<td>0.53</td>
<td>0.33-0.86</td>
<td></td>
</tr>
<tr>
<td>Chalkley count-</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>&lt; median</td>
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<td>0.02</td>
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<tr>
<td>&gt; median</td>
<td>1.70</td>
<td>1.09-2.66</td>
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</tr>
<tr>
<td>stage-</td>
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</tr>
<tr>
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<td>0.02</td>
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<td>1.05-2.93</td>
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</tr>
<tr>
<td>stage IIIa</td>
<td>1.95</td>
<td>1.19-3.42</td>
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</tbody>
</table>

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

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<th>HR</th>
<th>CI</th>
<th>p-value</th>
</tr>
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<td>tumour cell EGFR/MMP-9 expres-</td>
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<tr>
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<tr>
<td>positive</td>
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<tr>
<td>Bcl-2</td>
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</tr>
<tr>
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<td>0.005</td>
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<tr>
<td>positive</td>
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</tr>
<tr>
<td>Chalkley count-</td>
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</tr>
<tr>
<td>&lt; median</td>
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</tr>
<tr>
<td>stage I</td>
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<td>0.02</td>
</tr>
<tr>
<td>stage II</td>
<td>1.69</td>
<td>1.01-2.81</td>
<td></td>
</tr>
<tr>
<td>stage IIIa</td>
<td>1.95</td>
<td>1.15-3.29</td>
<td></td>
</tr>
</tbody>
</table>
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

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

**Figure 5.3b:**
Chalkley count < median and MMP-9 tumour cell negative

B vs C p=0.02

**Figure 5.3c:**
Chalkley count > median or MMP-9 tumour cell positive

**Figure 5.3d:**
Chalkley count > median and MMP-9 tumour cell positive

B vs C p=0.02

Legend for figure 5.3a:
A Chalkley count < median, MMP-9 tumour -ve
B Chalkley count > median or MMP-9 tumour +ve
C Chalkley count > median, MMP-9 tumour +ve

Legend for figures 5.3b, 5.3c, 5.3d:
Chalkley count < median, MMP-9 tumour -ve, Bcl-2 +ve
Chalkley count < median, MMP-9 tumour -ve, Bcl-2 -ve
Chalkley count > median or MMP-9 tumour +ve, Bcl-2 +ve
Chalkley count > median or MMP-9 tumour +ve, Bcl-2 -ve
Chalkley count > median, MMP-9 tumour +ve, Bcl-2 +ve
Chalkley count > median, MMP-9 tumour +ve, Bcl-2 -ve

13 cancer deaths/40 (33%)
46 cancer deaths/85 (54%)
30 cancer deaths/41 (73%)
1 cancer death / 10 (10%)
12 cancer deaths / 30 (40%)
14 cancer deaths / 33 (42%)
32 cancer deaths / 52 (62%)
10 cancer deaths / 16 (63%)
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

**Survival (days)**

- A: Chalkley count < median, EGFR/MMP-9 -ve
- B: Chalkley count > median or EGFR/MMP-9 +ve
- C: Chalkley count > median + EGFR/MMP-9 +ve

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

17 cancer deaths/52 (33%)
51 cancer deaths/87 (59%)
21 cancer deaths/27 (78%)

**figure 5.4b:**
Chalkley count < median and tumour cell EGFR/MMP-9 negative

**Survival (days)**

- Bcl-2 expression
  - positive
  - negative

**figure 5.4c:**
Chalkley count > median or tumour cell EGFR/MMP-9 expression

**Survival (days)**

- Bcl-2 expression
  - positive
  - negative

**figure 5.4d:**
Chalkley count > median and tumour cell EGFR/MMP-9 expression

**Survival (days)**

- Bcl-2 expression
  - positive
  - negative

p=0.02
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,
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™. 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.

<table>
<thead>
<tr>
<th>NSCLC cell-line</th>
<th>histology</th>
<th>EGFR immunointensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>H460</td>
<td>adenocarcinoma</td>
<td>+</td>
</tr>
<tr>
<td>A549</td>
<td>adenocarcinoma</td>
<td>+++</td>
</tr>
<tr>
<td>MORP</td>
<td>adenocarcinoma</td>
<td>+</td>
</tr>
<tr>
<td>SK-MES</td>
<td>squamous cell</td>
<td>+++</td>
</tr>
<tr>
<td>H647</td>
<td>adenosquamous</td>
<td>++</td>
</tr>
</tbody>
</table>

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)

- no EGF
- + 100nM EGF

100bp HT- PCR ladder 1080 control
H460 A549 MORP SK-MES H647 RT reaction

MMP-9 66°C 35 cycles (250bp)

- no EGF
- + 100nM EGF

100bp PCR ladder control H460 A549 MORP SK-MES H647 RT reaction

Figure 6.2 Immunohistochemistry for EGFR in H460 and A549 cell-lines.

a) H460 immunointensity score +

b) A549 immunointensity score +++
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)**

- no EGF
- + 100nM EGF

100bp PCR ladder 1080 control H460 A549 MORP SK-MES H647

**TIMP-1 59°C 30 cycles (350bp)**

- no EGF
- + 100nM EGF

100bp HT-PCR ladder 1080 control H460 A549 MORP SK-MES H647

**TIMP-2 59°C 30 cycles (250bp)**

- no EGF
- + 100nM EGF

100bp HT-PCR ladder 1080 control H460 A549 MORP SK-MES H647
Figure 6.4 Effects of EGF on the invasion index in NSCLC cell-lines.

<table>
<thead>
<tr>
<th>cell-line</th>
<th>Baseline invasion index</th>
<th>EGF-stimulated invasion index</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>invasion index 1</td>
<td>invasion index 2</td>
</tr>
<tr>
<td>H460</td>
<td>7.8</td>
<td>5.3</td>
</tr>
<tr>
<td>A549</td>
<td>18.9</td>
<td>13.2</td>
</tr>
<tr>
<td>MORP</td>
<td>0.5</td>
<td>1.8</td>
</tr>
<tr>
<td>SK-MES</td>
<td>23.4</td>
<td>27.6</td>
</tr>
<tr>
<td>H647</td>
<td>12.5</td>
<td>30.6</td>
</tr>
</tbody>
</table>

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

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 sequestered 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.
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) 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 α,β3 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 α,β3 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.
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.
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List of Publications Relating to Thesis

Original papers


Review articles


Submitted manuscript

Cox G, Jones JL, Andi A and O’Byrne KJ Molecular biological staging system for operable non-small cell lung cancer. Thorax

Invited review

Cox G, O’Byrne KJ. Matrix metalloproteinases in cancer. Anticancer Res

Associated papers


Published Abstracts


