Hepatocyte Growth Factor/Scatter Factor and *C-met* ligand-receptor in Human Breast Cancer

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This thesis is dedicated to my wife Maureen and our families.

"Nature never makes things for mean or no uses"

John Locke (1632-1704)

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List of abbreviations

ARG	arginine
BSA	bovine serum albumin
cDNA	complimentary deoxyribonucleic acid
CSF	colony stimulating factor
DAG	diacylglycerol
DNA	deoxyribonucleic acid
ECM	extracellular membrane
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
FAK	focal adhesion kinase
FGF	fibroblast growth factor
GAP	GTPase activating protein
GLA	gamma linolenic acid
GTP	guanine triphosphate
H&E	haematoxylin and eosin
HGF	hepatocyte growth factor
HGFA	hepatocyte growth factor activator
IGF	insulin-like growth factor
IHC	immunohistochemistry
IL	interleukin
IP	inositol triphosphate
LOH	loss of heterozygosity
MAP	mitogen activated protein
MDCK	Madin Darby canine kidney
mRNA	messenger ribonucleic acid
MVD	microvessel density
NGF	nerve growth factor
OD	optical density
PBS	phosphate buffered saline
PDGF	platelet derived growth factor

PI	phosphoinositide
PIP	phosphatidylinositol-4,5-P_2
PKC	protein kinase C
PLC	phospholipase C
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
SDS	sodium dodecyl sulphate
SF	scatter factor
SF-IF	scatter factor inducing factor
SSC	saline sodium citrate
STET	sucrose tris EDTA triton
TE	tris-EDTA
TEMED	N,N,N', N'-tetramethylethylenediamine
TBS	tris buffered saline
TGF	transforming growth factor
TNF	tumour necrosis factor
tPA	tissue plasminogen activator
TSP	thrombospondin
TVV	tumour vascular volume
uPA	urokinase plasminogen activator
VAL	valine
VEGF	vascular endothelial growth factor

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Nagy, J., Curry, G.W., McKay, I.C., Hillan, K.J., Purushotham, A.D., George, W.D. (1996) Hepatocyte growth factor/scatter factor expression and *c-met* in primary breast cancer. Surgical Oncology, 5, 15-21.

Nagy, J., Curry, G.W., Hillan, K.J., Mallon, E.A., Purushotham, A.D., George, W.D. (1996) Hepatocyte growth factor/scatter factor, angiogenesis and tumour cell proliferation in primary breast cancer. The Breast, 5, 105-109.

Cooke, A., Kim, Y.T., Harvey, T.I., Connor, J.M., Nagy, J., George, W.D. (1993) Loss of heterozygosity on chromosome 7q31 in breast cancer. Lancet, 341, 1289 (letter).

Published abstracts

Nagy, J., Clarke, J.S., Campbell, A., George, W.D. (1994) *C-met* expression in breast cancer. Scottish Medical Journal, 39, 60.

Nagy, J., Clarke, J.S., Cooke, A., Campbell, A., Connor, J.M., Purushotham, A.D., George, W.D. (1994) Evidence of overexpression but not loss of heterozygosity of the *c-met* gene in primary breast cancer. British Journal of Surgery, 81, 764.

Nagy, J., Hillan, K.J., Purushotham, A.D., George, W.D. (1994) Relationship of *c-met* receptor expression with clinical and pathological variables in breast cancer. Journal of Pathology, 173, Supplement, 7.

Nagy, J., Hillan, K.J., Purushotham, A.D., George, W.D. (1994) *C-met* expression in breast cancer. Clinical and Experimental Metastasis, 12, 52.

Nagy, J., Curry, G.W., McKay, I.C., Hillan, K.J., Purushotham, A.D., George, W.D. (1995) Hepatocyte Growth Factor/Scatter Factor in Breast cancer. British Journal of Surgery, 82, 699.

Nagy, J., Curry, G.W., Hillan, K.J., Purushotham, A.D., George, W.D. (1995) Hepatocyte Growth Factor/Scatter Factor and Tumour Angiogenesis in Primary Breast Cancer. British Journal of Surgery, 82, 1572.

Nagy, J., Curry, G.W., McKay, I.C., Hillan, K.J., Purushotham, A.D., George, W.D. (1995) Hepatocyte Growth Factor/Scatter Factor and *c-met* Expression in Primary Breast Cancer. British Journal of Cancer, 72, suppl XXV, 24.

Presentations to scientific meetings

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Nagy, J., Clarke, J.S., Cooke, A., Campbell, A., Connor, J.M., Purushotham, A.D., George, W.D. Evidence of overexpression but not loss of heterozygosity of the *c-met* gene in primary breast cancer. 79th Meeting of Surgical Research Society, 6-7 January 1994.

Nagy, J., Hillan, K.J., Purushotham, A.D., George, W.D. Relationship of *c-met* receptor expression with clinical and pathological variables in breast cancer. 169th Meeting of Pathological Society of Great Britain and Ireland, 6-8 July 1994.

Nagy, J., Hillan, K.J., Purushotham, A.D., George, W.D. *C-met* expression in breast cancer. Fifth International Congress of the Metastasis Research Society, National Institute of Health, Bethesda, Washington, USA, 28-30 September 1994.

Nagy, J., Curry, G.W., McKay, I.C., Hillan, K.J., Purushotham, A.D., George, W.D. Hepatocyte Growth Factor/Scatter Factor in Breast Cancer. 81st Meeting of Surgical Research Society, 5-6 January 1995.

Nagy, J., Curry, G.W., Hillan, K.J., Purushotham, A.D., George, W.D. Hepatocyte Growth Factor/Scatter Factor and Tumour Angiogenesis in Primary Breast Cancer. 82nd Meeting of Surgical Research Society, 6-7 July 1995.

Nagy, J., Curry, G.W., McKay, I.C., Hillan, K.J., Purushotham, A.D., George, W.D. Hepatocyte Growth Factor/Scatter Factor and *c-met* Expression in Primary Breast Cancer. B.O.A. and B.A.S.O. Joint Meeting, 9-11 July 1995.

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Statement of collaboration

The design of this thesis was constructed by myself with advice from Mr. A.D. Purushotham, Dr. K.H. Hillan and Professor W.D. George.

Chapter 2. Investigation of the frequency of LOH of the *c-met* proto-oncogene in human breast cancer

This section of the thesis was performed at the Duncan Guthrie Institute of Medical Genetics, Glasgow. I was involved in DNA extraction and Southern blot electrophoresis of blood and breast cancer samples under the supervision of Dr. A. Cooke.

Chapter 3. HGF/SF-*c-met* ligand receptor system in human breast cancer

This section of the thesis was performed at the University Department of Pathology, Western Infirmary, Glasgow. All tissue processing and Western blot studies were performed by myself. *C-met* expression was independently assessed by Mrs T. McShane. ELISA analyses of HGF/SF expression was performed by Dr. G.W. Curry.

Chapter 4. HGF/SF, angiogenesis and tumour cell proliferation in primary breast cancer

This section of the thesis was performed at the University Department of Pathology, Western Infirmary, Glasgow. Tissue processing and histology were performed by myself with assistance from Mr. and Mrs. R. Ferrier. The presence of tumour in each sample was confirmed by Dr. K.H. Hillan. Determination of percentage tumour vascular volume, microvessel densities and KI-67 index were performed by myself.

Statistical analyses were performed by Dr. I. C. McKay, University Department of Pathology, Western Infirmary, Glasgow.

References cited in this text have been read by myself.

Summary

HGF/SF-*c-met* is involved in stromal-epithelial regulation of cellular processes such as growth, dissociation, motility and invasiveness in epithelial cells [6,313] and angiogenesis in endothelial cells[248]. When coordinated they appear to play an important role in the morphogenesis of epithelial tissues [19]. There is increasing evidence that HGF/SF-*c-met* is an essential regulating component of breast duct development [13,208,276,334]. Aberrant expression of HGF/SF-*c-met* may therefore be involved in biological mechanisms that promote invasion and metastasis in human cancers [115]. Loss of heterozygosity (LOH) at the *c-met* locus in breast cancer patients has been associated with disease-relapse and reduced survival [16].

The aims of this thesis were:

1. To investigate the frequency and significance of LOH of *c-met* in patients with breast cancer.
2. To investigate HGF/SF and *c-met* expression in patients with breast cancer.
3. To investigate the relationship of HGF/SF to tumour proliferation and angiogenesis in patients with breast cancer.

Investigation on frequency of loss of heterozygosity of the *c-met* proto-oncogene in human breast cancer

Aberrant HGF/SF-*c-met* function in breast cancer was first suggested by the observation of a high frequency of LOH at 7q31 (the *c-met* locus) in human breast cancer patients [16]. In this study we examined blood DNA derived from 111 patients with primary breast

cancer using the pMet H probe by Southern blotting. Of the 111 patients, 52 patients were heterozygous for the Taq 1 restriction fragment length polymorphism (RFLP) of 7.5 and 4.0 kb. Tumour DNA from these patients were then analysed for LOH at 7q31. Complete LOH at 7q31 was observed in 4% of cases only. The results of this study failed to confirm the reported high frequency of LOH of *c-met* and do not support the presence of a tumour suppressor gene at 7q31 in human breast cancer.

HGF/SF-*c-met* ligand receptor system in human breast cancer

In this study a statistical association between HGF/SF and *c-met* in relation to disease-relapse in patients with primary breast cancer was demonstrated. Furthermore, the data confirm the presence of increased HGF/SF levels in human breast cancer when compared to tumour-free breast tissue. HGF/SF levels demonstrated statistically significant associations with disease-relapse and death. These findings concord with three similar studies [331,332,335]. *C-met* protein was observed in all samples of tumour-free breast tissue and in a proportion of patients with breast cancer. Patients with detectable *c-met* demonstrated a significant association with disease relapse. This pattern of receptor expression and relationship to disease progression has subsequently been confirmed [86]. These observations provide strong support to the hypothesis that aberrant HGF/SF-*c-met* expression occurs in breast cancer.

HGF/SF, angiogenesis and tumour cell proliferation in primary breast cancer

In this study, a significant correlation between HGF/SF levels and tumour angiogenesis was observed in patients with breast cancer. Further evidence of a link between HGF/SF and tumour angiogenesis is provided by the observation of a direct relationship between HGF/SF and the endothelial cell marker, von Willebrand's factor, in breast cancer [335]. Also in this study, HGF/SF levels significantly correlated with KI-67 index and therefore tumour proliferation. HGF/SF is known to promote breast epithelial cell growth *in vitro* [115,208,252]. However, HGF/SF promotion of breast cancer *in vivo* appears to be related to angiogenesis rather than direct stimulation of cellular proliferation [147].

Conclusions

The results of this thesis support the hypothesis that aberrant HGF/SF-*c-met* expression occurs in human breast cancer and is related to disease progression. HGF/SF expression in breast cancer is related to both angiogenesis and tumour proliferation. The involvement of a tumour suppressor gene at the *c-met* locus is not confirmed.

Chapter 1: Introduction and Proposal for Thesis

Breast cancer is common, affecting one in 12 women and causes 21,000 deaths per annum in the UK [57]. The majority of these deaths will be due to metastatic disease. The metastatic pathway involves processes such as tumour cell separation, proteolysis of extracellular matrix (ECM), occupation of cleared ECM due to increased cell motility, and angiogenesis [162]. In developing organ systems, organised invasive growth of epithelium into stromal tissue is often dependent upon the inductive effect of mesenchyme. Parallels are therefore often made between epithelial-mesenchymal interactions during development and during carcinogenesis [26]. Factors that regulate these interactions may therefore be of importance in the biology, prognosis and treatment of breast cancer.

Molecular biology of mammary gland development

The initial development of the mammary gland involves both an interaction between an epithelial rudiment at the nipple and the underlying mesenchyme and a response to maternal hormones of pregnancy and lactation [53]. At birth, a primitive epithelial duct with few side branches has developed embedded in the mesenchymal fat pad. During puberty, ductal elongation and branching morphogenesis occur. The final stage of breast development, lobuloalveolar differentiation, occurs during pregnancy where multiple alveoli bud from ducts to form the milk secretory units. Development of the normal breast is regulated by endocrine hormonal action. Such hormones exert their effects

through local production of growth factors that interact in an autocrine or paracrine fashion. The hormonal regulation of breast development is summarised (Table 1.1). Inductive interactions between epithelium and mesenchyme are essential for growth and differentiation of the mammary gland during embryogenesis and throughout postnatal life (Figure 1.1). Branching morphogenesis is an important stage in the development of different epithelial organs and the adjacent mesenchyme appears to be of importance [259]. The inducing effect of the mesenchyme is partly due to interactions with the ECM [1,5]. However, culture of mammary gland epithelial cells in collagen gels allows only limited growth and branching morphogenesis suggesting additional signals from stromal cells are required [11,44,235,333]. The identity of soluble factors involved in stromal epithelial interactions during the development of the mammary gland are the subject of ongoing investigation. Epithelial tyrosine kinase receptors and their stromal ligands may be of importance. These ligand-receptor pairs were initially associated with mediating mitogenic signals because of their transforming potential. However, recent studies have demonstrated tyrosine kinase receptors can also regulate differentiation, cell movement and morphogenesis [18,187,188,265]. Furthermore, studies of the development of kidney, lung and liver in mice have supported the importance of mesenchymal ligands of epithelial tyrosine kinases [20,189,220,222,261,263,265,310].

Hepatocyte growth factor/scatter factor (HGF/SF) is a mesenchymal/stromal derived cytokine. The tyrosine kinase receptor to HGF/SF, encoded by the *c-met* proto-oncogene, is expressed in a wide variety of human epithelial cell types. Unlike many other growth factors such as the epidermal growth factor (EGF) family, transforming

Table 1.1

Sequence of regulatory factors in development of mammary gland (Dickson et al[53]).

Stage	Systemic factors	Local factors
Epithelial bud	(+)Transplacental hormones	Unknown local factors Stromal induction
Fat pad penetration	(+) Oestrogen (+) Growth hormone	(+?) TGF-α (+) IGF (-?) TGF-β
Ductal elongation, branching following puberty	(+) Oestrogen (+) Growth hormone (+) Progesterone (+) Prolactin (+) Insulin (+)Glucocorticoids	(+?) TGF-α (+) IGF (-?) TGF-β
Lobuloalveolar differentiation	(+) Oestrogen (+) Progesterone (+) Oxytocin (-?) Prolactin	(+?) TGF-α (+) EGF (+) CSF-1 (+?) FGF (-?) TGF-β

TGF transforming growth factor
IGF insulin-like growth factor
FGF fibroblast growth factor
EGF epidermal growth factor
CSF colony stimulating factor

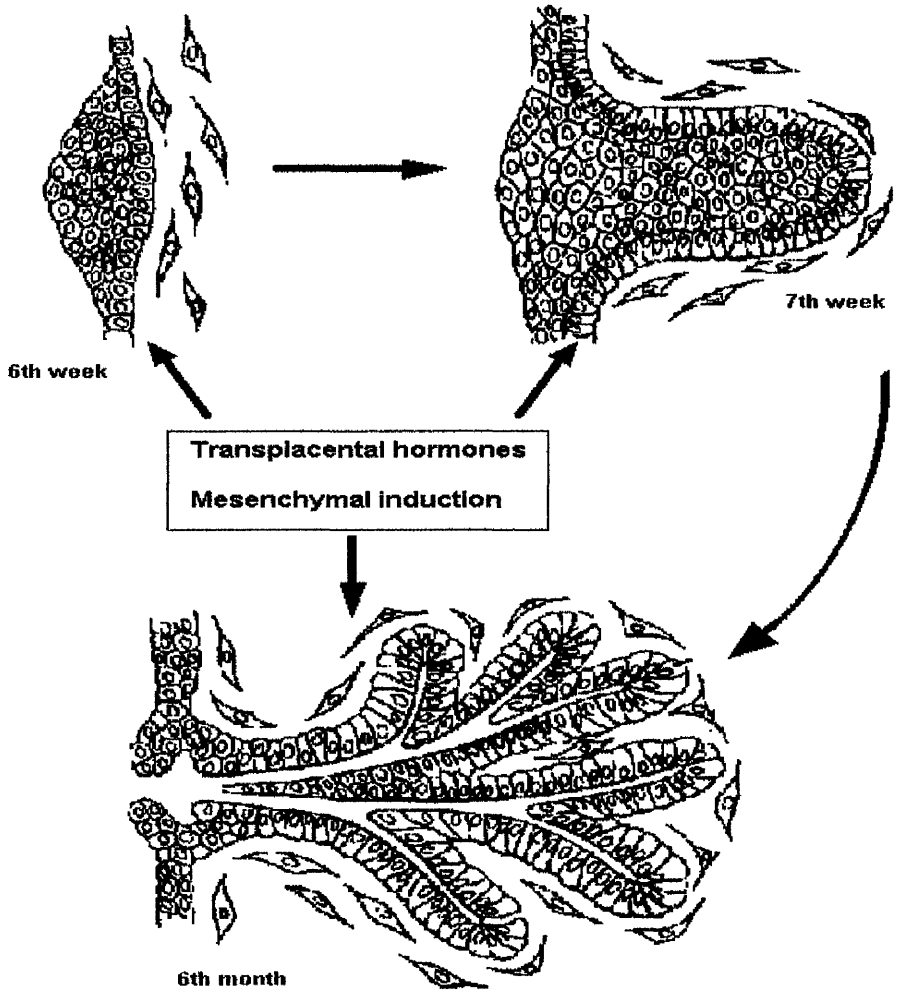


Figure 1.1

Development of human mammary gland. The mammary ridges first appear at 4 weeks as thickened lines of epidermis extending from thorax to thigh. Under the influence of transplacental hormones of pregnancy and inductive effect of mesenchyme, mammary ridge ectoderm proliferates in the 5th week to form primary mammary buds. Secondary buds form during the 3rd month and become canalised to form lactiferous ducts during the last 4 months of foetal life. Organisation of lactiferous ducts around the developing nipple occurs in the 8th month. Factors such as TGF-α, TGF-β and IGF may mediate hormone induced signals. The local mediators of mesenchymal induction are largely unknown.

growth factor (TGF)-β, fibroblast growth factor (FGF) 1 & 4, HGF/SF appears to act principally as paracrine effector of normal epithelial cells. There is evidence that HGF/SF-*c-met* may play a role in the stromal-epithelial interactions participating in morphogenesis. In collagen matrix, HGF/SF induces the formation of branched tubules in Madin-Darby canine kidney (MDCK) epithelial cells [187,188]. Furthermore, HGF/SF has been shown to induce lumen-like structures in malignant breast epithelial cells in culture [303]. The role of HGF/SF and *c-met* in organ morphogenesis is supported by in-situ hybridisation and RNase protection experiments where *c-met* is detected predominantly in epithelial cells of various developing organs whereas the ligand is expressed in adjacent stromal cells [275]. Aberrant expression of this ligand/receptor pair may result in cell dissociation, invasion and metastasis. LOH of *c-met* in human breast cancer patients has been associated with reduced disease-free and overall survival [16]. Furthermore, high levels of HGF/SF have been demonstrated in human breast cancer extracts [331]. The subject of this thesis is to further investigate HGF/SF and *c-met* in human breast cancer.

Review of HGF/SF and its receptor, the *c-met* proto-oncogene product

Discovery of HGF and scatter factor

HGF was identified as a blood borne polypeptide present in the bloodstream of hepatectomised animals and capable of stimulating hepatocyte proliferation [177,196,255]. In 1984, Stoker observed that human mammary epithelial cells cultured in fibroblast conditioned media appeared as contiguous cell sheets. When grown in media

conditioned by MRC 5 fibroblasts the cells were dissociated [279]. A similar effect with MDCK cells suggested that MRC 5 fibroblasts produce an epithelial scatter factor (SF) [282]. Sequence analysis of the N-terminal of the subunit of SF revealed it to be the corresponding portion of HGF [85]. By a combination of sequence, immunochemical and biological analysis it was established that HGF and SF were identical [75,85,142,319,320]. The high affinity receptor to HGF/SF was subsequently identified as the *c-met* proto-oncogene product [23,202].

Molecular structure of HGF/SF

HGF/SF has been fully purified, amino acid sequence determined and cDNA cloned. HGF/SF is a single chain peptide of 728 amino acid residues containing a 29 amino acid signal sequence and a 25 amino acid pro-sequence [182,198,340]. Synthesised and secreted as a single chain pro-form HGF/SF needs to be converted to the mature heterodimeric form by extracellular cleavage. The mature HGF/SF molecule is a disulphide linked heterodimeric protein consisting of a 69 kDa heavy α-chain and a 34kDa light β-chain (Figure 1.2) [91,197,339]. Molecular cloning and recombinant expression of HGF/SF reveal that it is secreted in the monomeric 90kDa form [179,252]. Biologic activity of HGF/SF requires the proteolytic hydrolysis of an Arg-Val bond within its prosequence by enzymes with serine protease activity [181,194,198,203,205,340]. Several enzymes, including urokinase plasminogen activator, have been shown to activate HGF/SF (Figure 1.2) [77,201]. Although monomeric HGF/SF binds to its receptor with high affinity, proteolytic processing into the heterodimeric form is required for mitogenic and scattering activity

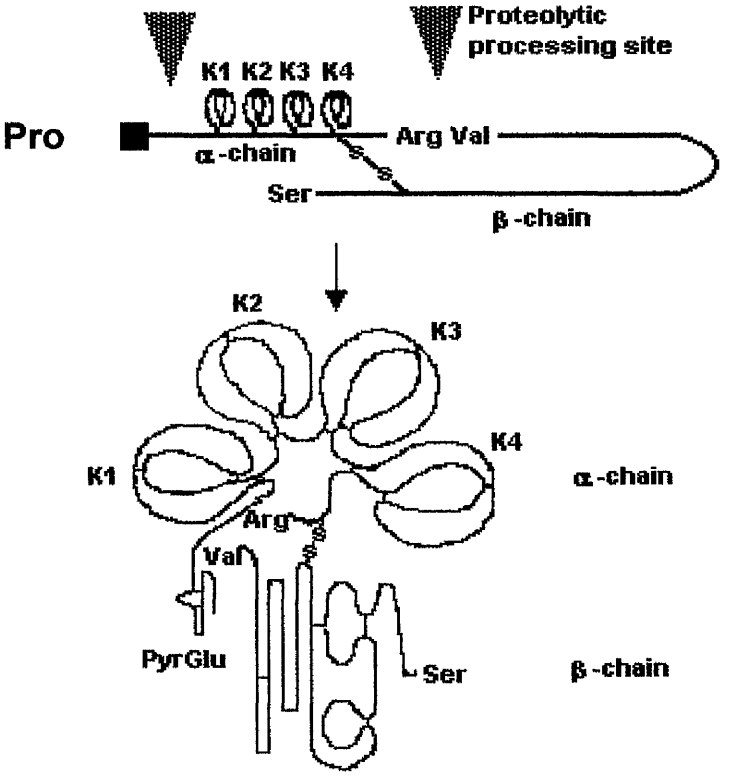


Figure 1.2

The mature HGF/SF molecule is a heterodimer consisting of a 69 kDa α-chain and a 34 kDa β-chain. Enzymatic hydrolysis of an arginine-valine (Arg-Val) bond within pro-HGF/SF by serine proteases produces the active heterodimeric form of HGF/SF. K1-K4 represent the four kringle domains of HGF/SF which are involved in receptor binding and ligand activity.

[77,158,185,193,201]. The α-chain contains 4 kringle domains that are essential for protein-protein interactions [174]. Mutagenesis and deletional studies have shown that kringles 1 and 2 are essential for receptor binding, whereas kringles 3 and 4 are needed for biological activity [174,184]. The HGF/SF gene has been localised by in-situ hybridisation with a 3H-labelled probe to chromosome 7q11.2-21 [257,319].

Source of HGF/SF

HGF/SF production has been detected in numerous tissues including fibroblasts, endothelial cells, Kupffer cells and lipid-storing cells of the liver [122]. In addition tumour cells have been observed to express HGF/SF mRNA or produce HGF/SF protein [102,178,256,280,337]. Serum HGF/SF concentrations are also elevated in patients with liver disease and in some cancer patients [293,294,305]. Tanaguchi *et al*, observed a decrease in serum HGF/SF levels following breast cancer surgery suggesting tumour as the primary source [294].

HGF/SF is expressed by many organs throughout the body. It has been detected at the mRNA or protein level in cultured fibroblasts derived from organs such as lung, stomach, colon, breast, prostate and skin [252]. Tissue extraction and immunohistochemical (IHC) studies initially demonstrated expression in the pancreas, small intestine, salivary glands, thyroid and brain [341]. Further studies confirmed these findings and also detected strong signals in normal human breast and other tissues (Table 1.2) [48,306,328,329].

Table 1.2

Immunoreactivity for HGF/SF in human and rat tissues (Wolf *et al* [328]).

Strong	Moderate	Weak	Negative
Human breast	Lung	Adenohypophysis	Parathyroid
Skin	Gastrointestinal tract	Adrenal cortex	Adrenal medulla
Bladder	Salivary glands	Thyroid	Lymphocytes
Mesothelium	Exocrine pancreas	Testis	Myocytes
Prostate and seminal vesicles	Biliary tree	Proximal renal tubules	Glial cells
Distal renal tubules	Uterus and fallopian tubes		
Collecting ducts	Endothelium		
Syncitiotrophoblast	Macrophages		
Extravillous trophoblast			
Large neurones			
Megakaryocytes			
Granulocytes			

Autocrine and paracrine action of HGF/SF

There has been extensive investigation into whether HGF/SF exerts its function by autocrine or paracrine pathways. Studies both *in vitro* and *in vivo* suggest that HGF/SF is a mesenchymal/stromal derived cytokine serving as a paracrine effector of stromal-epithelial interactions. HGF/SF production *in vitro* appears to be restricted to predominantly fibroblast and not epithelial cell lines [280]. Other mesenchymal cells have been reported to produce HGF/SF, for example: Kupffer cells, endothelial cells, lipid-storing cells in the liver, endothelial cells in the lung, mammary adipocytes and HL-60 myelo-monocytic cells [172,210,230,233,271,280]. During development, HGF/SF is expressed in distinct mesenchymal embryonic tissues lying in close proximity to *c-met* expressing epithelium, suggesting a paracrine mode of interaction [275,304]. Furthermore, the HGF/SF gene promoter is active in mesenchymal but not epithelial cells supporting a paracrine model of stromal-epithelial interaction [224].

Studies on certain cancer cell lines suggest that HGF/SF and *c-met* may also interact in an autocrine fashion [54,56]. The gastric cancer cell line MKN 45 expresses both HGF/SF and *c-met* [76]. Co-expression of HGF/SF and *c-met* has also been detected in pancreatic cancer, peripheral nerve sheath tumour and gliomal cells [22,63,149,234]. In addition, HGF/SF expression has been observed in both epithelial and stromal cells of breast cancer and gastro-intestinal tissues [307,317,318]. Although HGF/SF is primarily thought to act in a paracrine fashion this may not be true for tumour cells. This conclusion is of importance as *in vitro* generated HGF/SF autocrine loops promote both tumour invasion

and metastasis *in vivo* [115].

Regulation of HGF/SF production

Fibroblast production of HGF/SF *in vitro* has been shown to be regulated by various agents, many being inflammatory cytokines. Interleukin 1α (IL-1α), IL-1β, tumour necrosis factor-α (TNF-α), EGF, platelet-derived growth factor (PDGF), FGF and prostaglandins have been shown to increase HGF/SF expression (Figure 1.3) [90,170,171,292]. Oestrogen has been reported to increase HGF/SF gene transcription in the mouse ovary [157]. TGF-β and glucocorticoids block HGF/SF induction by IL-1 and other stimuli [170, 233]. The 5'-flanking region of the HGF/SF gene contain sequences homologous to responding elements of p53, retinoblastoma protein (Rb), IL-1, IL-6, glucocorticoids and TGF-β [3]. Injurin, a humoral factor that is produced in non-injured distant organs following hepatic or renal injury, induces HGF/SF mRNA in rat lungs [173]. Recently a second factor with HGF/SF converting effects has been characterised and termed hepatocyte growth factor activator (HGFA) which is activated exclusively by injured tissues [180].

Co-culture experiments have indicated several possible *in vivo* mechanisms of HGF/SF regulation by epithelial cells. HGF/SF transcript levels in MRC 5 fibroblasts were initially reported to be decreased by co-culture with a human keratinocyte cell line through transcriptional regulation [130]. Similarly, Seslar *et al* observed inhibition of HGF/SF expression in MRC 5 fibroblasts when co-cultured with several human breast

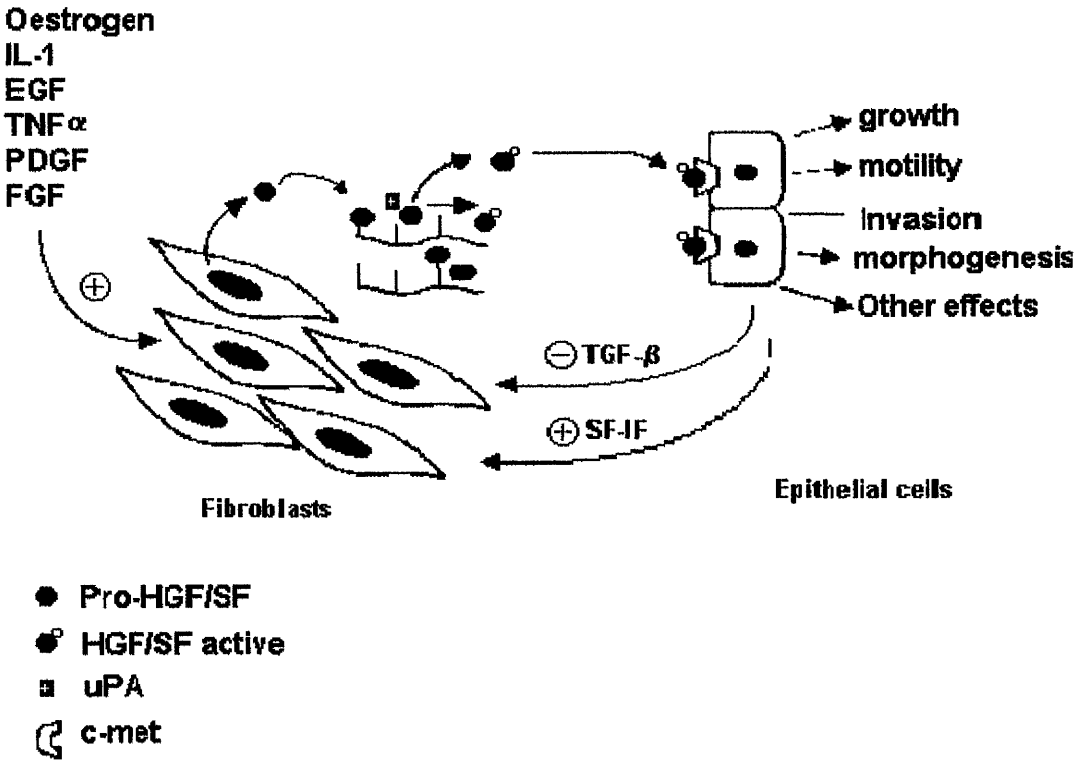


Figure 1.3

Schematic illustration of activation, regulation and actions of HGF/SF on epithelial cells. HGF/SF is produced as inactive pro-HGF/SF that is proteolytically processed in extracellular stroma by urokinase plasminogen activator (uPA). Fibroblast production of HGF/SF is regulated by epithelial cells through SF-IF and TGF-β. Other factors such as oestrogen and inflammatory cytokines also promote HGF/SF production.

carcinoma cell lines [268]. In addition, the conditioned medium collected from these cells stimulated HGF/SF production. These findings indicate that epithelial cells produce factors that inhibit or stimulate fibroblast production of HGF/SF. Subsequent work identified the negative regulator as TGF-β [267]. The protein present in conditioned media responsible for inducing HGF/SF production has been identified as scatter factor-inducing factor (SF-IF), a 14 kDa heat stable protein [246].

HGF/SF expression appears to be density dependent *in vitro* [267]. Mesenchymal condensations, necessary for normal morphogenesis of overlying epithelium, demonstrate high levels of HGF/SF expression in mouse embryos [101,275]. Similarly, desmoplasia is often observed adjacent to invading cancer cells [37,311]. In this context, HGF/SF immunostaining in cancer tissues is concentrated at areas of tumour invasion [338].

The HGF/SF receptor: *c-met*

The receptor to HGF/SF, encoded by *c-met*, was first identified as an activated oncogene in the N-methyl-N'-nitro-N-nitrosoguanidine treated human osteosarcoma cell line (MNNG-HOS) by its ability to transform NIH 3T3 fibroblasts [39]. The identity of the HGF/SF receptor was suggested by the observation that it stimulated the tyrosine phosphorylation of a 145 kDa protein [252]. The molecular weight and ligand specificity suggested that it might represent a growth factor receptor kinase. The use of antibodies specific for different tyrosine kinases identified the 145 kDa phosphoprotein as the β-subunit of the *c-met* proto-oncogene [23]. Cross linking experiments established the *c-met*

protein as the HGF/SF receptor [23,206]. Furthermore, kinase activation and cross-linking to the *c-met* protein was observed in a variety of cells that responded to HGF/SF [23,100,202,205]. Transfection of MDCK cells with a hybrid cDNA encoding the ligand binding domain of a nerve growth factor (NGF) receptor, *trk*, with membrane and cytoplasmic domains of *c-met* conferred NGF-responsiveness in motility, proliferation and morphogenic assays [321]. The pleiotropic effects of HGF/SF therefore appear to be mediated by one receptor [141,321]. The mature form of the receptor is a heterodimer consisting of a 45-50 kDa extracellular α-subunit linked via disulphide bonds to a 140-145 kDa β-subunit that contains extracellular, transmembrane and intracellular catalytic domains (Figure 1.4) [88,297]. The *c-met* gene has been mapped to chromosome 7 at q21-31 and encodes a 1408 amino acid glycoprotein which lacks significant homology to any other growth factor receptor [216]. *C-met* has been found to belong to a family which includes proto-oncogenes *Ron* and *c-sea* [81,226,241].

HGF/SF receptor expression and regulation

C-met is expressed by breast epithelial cells and a wide range of other epithelial cells [26,118,303]. Vascular endothelium is also known to express *c-met* [25]. The receptor is primarily located at intercellular junctions in conjunction with cell adhesion molecules such as E-cadherin [43]. Ligand binding of the *c-met* protein is associated with the activation of its catalytic activity [204]. The major autophosphorylation site is Y1235 which corresponds to the primary autophosphorylation site of other tyrosine kinases [70]. *C-met*-protein levels in hepatocyte cell cultures appear to be influenced by cell density.

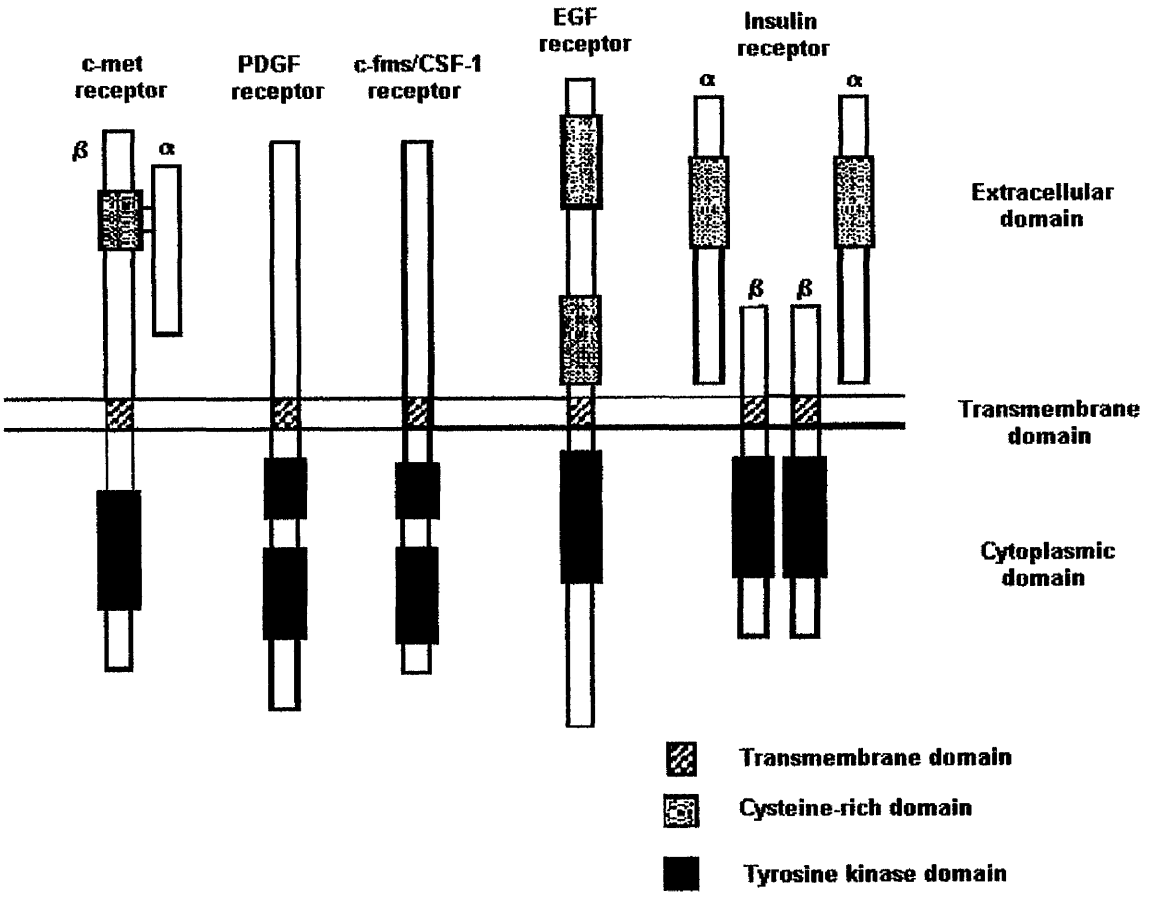


Figure 1.4

Schematic comparison showing several growth factor receptors containing intracellular tyrosine kinase domains.

Confluent cells express low levels, whereas at low density higher expression is found [21,183]. *C-met* mRNA levels are strongly upregulated by the cytokines IL-1α, IL-6, TNF-α, and TGF-β in human breast, ovarian and endometrial carcinoma cell lines [186]. Furthermore, steroidal hormones such as oestrogen, progesterone, and tamoxifen have similar effects on certain ovarian and endometrial cancer cell lines [186]. *C-met* mRNA levels undergo continuous turnover with half lives of less than 30 minutes, suggesting that *c-met* may behave as an immediate response gene [21,186]. An increased level of *c-met* mRNA has also been detected following cell stimulation with nanomolar concentrations of HGF/SF [21]. This observation suggests that cell response to HGF/SF may be autoamplified by inducing its specific receptor.

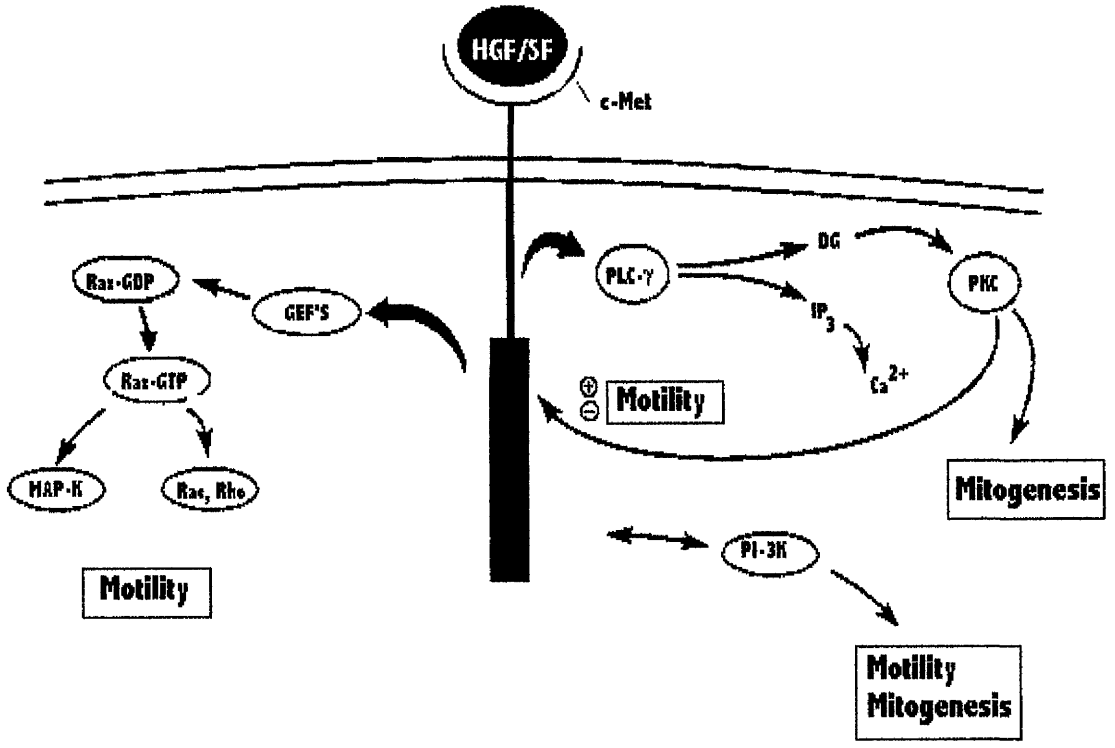
Post receptor signal transduction

The pleiotropic responses induced by HGF/SF occur through the one receptor encoded by *c-met* [89,188,309,320,321,349]. The molecular basis for these divergent responses is not fully understood. Protein engineering experiments indicate that mitogenic and motogenic responses diverge at the post receptor level [84]. Such diverse responses are therefore likely to result from the integration of several transductional pathways activated by coupling of the HGF/SF receptor to cytoplasmic transducers. Interactions with other cytokines and ECM molecules may also modulate the type of response.

Ligand binding to tyrosine kinase growth factor receptors results in dimerisation and autophosphorylation. However, *c-met* appears to form non-covalent clusters on cell

membranes *in vitro* suggesting that multimersisation may be required for signal transduction [67]. The HGF/SF receptor, when autophosphorylated, binds to a number of substrates containing Src homology 2 domains (SH2) such as phosphoinositide 3-kinase (PI 3-kinase), Ras-guanine nucleotide activating protein (Ras-GAP); phospholipase C-γ (PLC-γ) , pp60src and the GRB-2-Sos complex (Figure 1.5) [8,66,95,225,226]. The cytoplasmic domain contains specific tyrosine phosphorylation sites (Y1234 and Y1235) that positively regulate the kinase activity of the receptor [70,159,204]. Phosphorylation of Serine 985 has been shown to negatively regulate kinase activity [78]. Tyrosines 1347 and 1345 of the HGF/SF receptor form a docking site that mediate direct interactions with PI-3 kinase, PLC-γ, pp60src and GRB-2-Sos [226]. Particular tyrosine residues in the intracellular domain of the *c-met* protein have specific properties. Tyrosine 1356 appears to associate with GRB-2 protein and is essential for the transduction of HGF/SF induced motility and morphogenesis in MDCK cells [348]. Tyrosines 1347 and 1354 are also associated with both HGF/SF induced motility and morphogenesis in MDCK cells [322].

Phosphorylation of PLC-γ has been detected following *c-met* activation [214]. This results in the hydrolysis of phosphatidylinositol-4,5-P_2 (PIP_2) to form diacylglycerol (DAG) and inositol triphosphate (IP_3). DAG and IP_3 stimulate distinct downstream signalling pathways, protein kinase C (PKC) and calcium mobilisation. The activation of PKC by HGF/SF-*c-met* interaction can result in stimulation of MAP (mitogen activated protein) kinase pathways but can also result in negative regulation of *c-met* activity [122]. PLC-γ activation is necessary for cell motility and is related to the mobilisation of several



PLC	Phospholipase C
DG	Diacylglycerol
IP3	Inositol triphosphate
GEF	Guanine nucleotide exchange factors
MAP-K	MAP-kinase
PI-3K	PI-3kinase

Figure 1.5

HGF/SF-*c-met* signal transduction pathways. HGF/SF binds to and induces tyrosine phosphorylation of the *c-met* receptor. This promotes binding of intracellular signalling proteins containing *src* homology (SH2) regions such as PLC-γ, guanine nucleotide exchange factors and PI-3 kinase. Each SH2-containing protein may activate a different subset of signalling phosphopeptides thus eliciting different responses within the cell.

actin-modifying proteins such as gelsolin and profilin [34]. *Ras, rho, raf* and *rac* proteins are also involved in *c-met* signalling. Activation of *c-met* results in activation of Ras-GAP which in turn interacts with effector proteins including the serine/threonine kinase, *raf* [218]. Growth factors induce alterations in cell movement and cell shape by remodelling the actin cytoskeleton. Such alterations are mediated by the *rho* subfamily (*rho, rac* and cdc42) of small guanine nucleotide binding proteins. HGF/SF activates *rho* and its associated proteins which in turn induce alterations in the actin framework [122].

Biological activities of HGF/SF and *c-met*

From its initial discovery, HGF/SF has been implicated in the processes that regulate tumour cell behaviour. HGF/SF has been shown to induce cell growth, motility and invasiveness that play a physiological role in development. However, these processes are similar to those promoting tumour invasion and metastasis.

HGF/SF regulates motility and growth of cancer cells

HGF/SF stimulates motility and growth of many tumour cell types although the degree of response may vary. Three types of response have been observed: increased proliferation and motility; decreased proliferation and motility; no effect on motility but varied effects on proliferation. The type of response observed is dependent on tumour cell type [122]. HGF/SF and *c-met* may be involved in cell cycle regulation. *C-met*

expression has also been observed to be cell cycle dependent, the level of expression being highest at G2M and decreasing from S phase to G0/G1 [156]. HGF/SF stimulates DNA synthesis/proliferation in a wide range of epithelial cells including mammary epithelial cells, hepatocytes, biliary epithelial cells, keratinocytes, melanocytes, melanoma cells, gastric, colonic and lung cancer cells, and vascular endothelial cells [25,91,98,128,167,197,252,269,285,339]. *C-met* has been found to be associated with Bag-1, a protein involved in mechanisms that prolong cell survival. The formation of Bag-1-*c-met* complexes increases rapidly following the induction of apoptosis [7]. This suggests that HGF/SF-*c-met* may be involved in both regulation of cell growth and death.

HGF/SF and cancer invasive/metastatic behaviour

The metastatic cascade is composed of separate steps: loss of cell-cell adhesion; adhesion followed by invasion of basement membrane and ECM; intravasation, travel and survival in circulation; adhesion to endothelium followed by extravasation; formation of secondary foci supported by angiogenesis. HGF/SF has been implicated in several of these processes.

HGF/SF, cell-cell adhesion and motility

The dissociation/motility promoting properties of HGF/SF were described by Stoker *et al* on human mammary epithelial cells *in vitro* [279]. Increased motility and migration has been seen in several cancer cell types including breast [42,123,125,274,280].

Following stimulation of tumour cell colonies with HGF/SF, membrane ruffling is seen preceding dissociation [118,280,281]. Intercellular cell-cell contacts are mediated by cell surface adhesion molecules. There is evidence for HGF/SF inhibiting cell adhesion systems. HGF/SF treated cells fail to form stable cell-cell adhesion junctions through increased stability of newly synthesised soluble E-cadherin and altered phosphorylation of E-cadherin within the cell [217]. Cadherin function is modulated by intracellular proteins named catenins. HGF/SF has been shown to enhance the tyrosine phosphorylation status of β-catenin which in turn down regulates cadherin mediated cell adhesion [105,270,295]. Thus HGF/SF promotes dismantling of cell-cell adhesion complexes and inhibits the formation of adherens junctions [123]. The HGF/SF receptor, *c-met*, is located at intercellular junctions together with cell adhesion molecules such as E-cadherin [43]. However, whether this localisation pattern facilitates physical interactions between these molecules is unknown.

HGF/SF, cell-matrix adhesion, matrix degradation and invasion

The basement membrane and ECM form the first barrier that tumour cells encounter through the metastatic process. Tumour cells adhere to ECM via the integrin family of cell-adhesion molecules. Integrins in turn interact with intracellular cytoskeletal elements. Following integrin binding, focal adhesions are formed consisting of integrins, p125 FAK (focal adhesion kinase) and paxilin. During malignant transformation, changes in integrin expression have been observed and may contribute to anchorage-independent growth and invasion [47,87]. HGF/SF increases adhesion of *c-*

met positive lymphoma cells to fibronectin and promotes migration and invasion [326]. HGF/SF has been shown to increase integrin expression in both breast and liver cancer cells thus increasing their matrix adhesive properties [133,206]. HGF/SF also affects the initial recruitment of integrins, cytoskeletal proteins, p125 FAK and paxilin into focal adhesion complexes via *c-met* [123,169].

Following the adhesion to ECM, tumour cells must invade this barrier. HGF/SF induces the invasion of a number of epithelial cells through collagen matrices and basement membranes [10,15,115,169,244,247,274,283,320]. The formation of HGF/SF-*c-met* autocrine loops have been shown to promote tumour cell invasiveness in NIH 3T3, SK-LMS-1 and C127 breast cancer cells [114]. Invasion of ECM components require the action of activated proteolytic enzymes. The tpr-met hybrid is known to activate the urokinase plasminogen activator (uPA) gene [14]. The production of uPA and collagenase are increased by HGF/SF [219,247]. These proteases may be involved both in ECM degradation and activation of HGF/SF. HGF/SF also stimulates cancer cell production of matrix metalloproteinases, collagenase-1 and stromelysin-1 [12,61] .

To gain access to the circulation, tumour cells must bind to endothelial cells. HGF/SF has been shown to increase the adhesion of tumour cells to endothelium. HGF/SF increases expression of integrins α-2 which promotes tumour cell adhesion to matrix and to endothelial cell substrates [133]. HGF/SF also increases the expression of CD44 on endothelial cells, a molecule which may be involved in tumour-endothelial interactions and establishment of metastases [104]. Melanoma cells with high *c-met* and HGF/SF

activity demonstrate increased liver colonisation [254].

HGF/SF and morphogenesis

The ruffling of free edges on the apical surface of cells is one of the earliest morphological events to occur following HGF/SF stimulation [58,124]. Montesano *et al* observed MDCK cells forming organised tubular structures when grown in collagen gels in the presence of HGF/SF obtained from fibroblast conditioned medium or when grown with fibroblasts [187,188]. Tsarfaty *et al* demonstrated that HGF/SF induced human breast and colonic carcinoma cells to form lumen like structures *in vitro* [303]. Both non-parenchymal liver epithelial cells and prostate epithelial cells in collagen matrices demonstrate branching morphogenesis when stimulated by HGF/SF [122,127]. Similar morphoregulatory properties in epithelial cells have not been observed to the same degree by other growth factors such as EGF, basic FGF, TGF-β, insulin-like growth factor (IGF) or PDGF [13,276]. HGF/SF has also been reported to stimulate the formation of tubular structures by vascular endothelial cells *in vitro* and angiogenesis *in vivo* [25,94,190,245]. *In vivo*, HGF/SF has been shown to generate axial structures in chick embryos [278]. HGF/SF-*c-met* is an important mediator of limb and hepatic development [20,263]. Mice lacking HGF/SF demonstrate placental defects and embryonic lethality [310].

HGF/SF and angiogenesis

Angiogenesis is a prime requirement for tumour growth and metastasis. Angiogenesis requires a number of features such as proliferation and migration of endothelial cells and appropriate regulation of endothelial cell-cell and cell-matrix adhesion. HGF/SF stimulates endothelial cell proliferation *in vitro* [25,94]. *In vivo* studies also demonstrate HGF/SF to be a powerful inducer of angiogenesis in rodents [25,94,243]. The angiogenic promoting properties of HGF/SF are amplified by tissue plasminogen activator (tPA) and vascular endothelial growth factor (VEGF) [122]. HGF/SF induced angiogenesis may occur by autocrine or paracrine mechanisms as both HGF/SF and *c-met* are expressed in endothelial and vascular smooth muscle cells [199,200]. Glioma cells produce active HGF/SF which stimulates angiogenesis in a paracrine fashion [147,149]. Similarly, breast cancer cells transfected with HGF/SF are able to stimulate angiogenesis as part of a mechanism of increased tumorigenicity[147].

HGF/SF in tumour-stroma interaction

Interactions between epithelium and mesenchyme mediate important aspects of normal growth, morphogenesis and neoplasia. The growth, differentiation and morphogenesis of developing epithelia are regulated by the neighbouring mesenchyme of various organs [18,260]. During development, HGF/SF is expressed predominantly in mesenchymal cells whereas *c-met* expressed in cells of epithelial origin [275]. HGF/SF induces and organises branching tubulogenesis of kidney, lung and mammary gland

[187,208,213,262,276,334]. Thus, during organogenesis HGF/SF plays an important role as a mesenchymal derived mediator of organised cell growth, movement, morphogenesis and ECM remodelling.

Stromal influence on epithelial neoplasia and malignant progression of carcinoma cells has been reported in a number of cancers including breast cancer [18,260,312]. The growth of certain carcinoma cells *in vivo* is accelerated by fibroblasts. Furthermore, *in vitro* and *in vivo* invasiveness of several carcinoma cells are induced by the co-existence of stromal fibroblasts [29,96,168,221]. Tumour cell invasion and metastases may therefore be driven by a number of stromal-derived factors. Although the molecular mechanisms involved in tumour-stroma interactions are not fully understood, HGF/SF may be involved.

HGF/SF has been identified as a fibroblast-derived invasion factor *in vitro*. Most carcinoma cells *in vitro* do not invade ECM e.g. collagen gels [83,168,264]. Invasion of oral squamous cell carcinoma cells has been shown to be induced by incorporation of normal fibroblasts or conditioned medium into collagen gels [168]. The factor responsible for inducing this invasiveness was identified as HGF/SF [169]. Subsequently Weidner *et al* demonstrated HGF/SF induced invasion and migration of a wide variety of tumour cells [320].

HGF/SF production may be stimulated by tumour-derived inducers. Human breast cancer cells are able to regulate fibroblast production of HGF/SF by SF-IF and TGF-β

[246,268]. Additional HGF/SF inducers have been shown to be produced by tumour including: IL-1α, IL-1β, basic FGF, PDGF and prostaglandin [170,173,195]. These cytokines and growth factors transcriptionally activate gene expression of HGF/SF [90,171,174,195,238].

Tumour progression may involve the presence of a HGF/SF inducer loop. Tumour cells appear to produce factors that induce fibroblast production of HGF/SF and fibroblasts then secrete HGF/SF which stimulates invasive growth. Co-culture experiments have demonstrated the existence of these interactions *in vitro*. Of importance, in these experiments invasion was inhibited by both antibodies against HGF/SF and inhibitors of HGF/SF inducers [165,166,170,195]. The stromal matrix may act as a reservoir for HGF/SF. HGF/SF binds to several matrix proteins including thrombospondin-1 (TSP-1), fibronectin, laminin, collagen type 1, heparan sulphate and basement membrane [131,148,161]. Matrix bound HGF/SF is still able to exert biological functions [131].

HGF/SF and tumour metastasis

HGF/SF-*c-met* autocrine stimulation has been observed to promote tumour invasion and metastasis *in vivo* (Figure 1.6). NIH 3T3 cells transfected with *c-met* exhibit a high frequency of lung metastases compared with control cells when implanted in nude mice [115]. Furthermore, HGF/SF-*c-met* signalling facilitated NIH 3T3 cells to colonise organs such as lung, heart, diaphragm and retroperitoneum [115]. Similar findings were observed in C127 breast cancer and SK-LMS-1 leiomyosarcoma cells [115]. *In vitro* treatment of the *c-met* expressing EMT6 mouse mammary tumour cell line with HGF/SF has also been

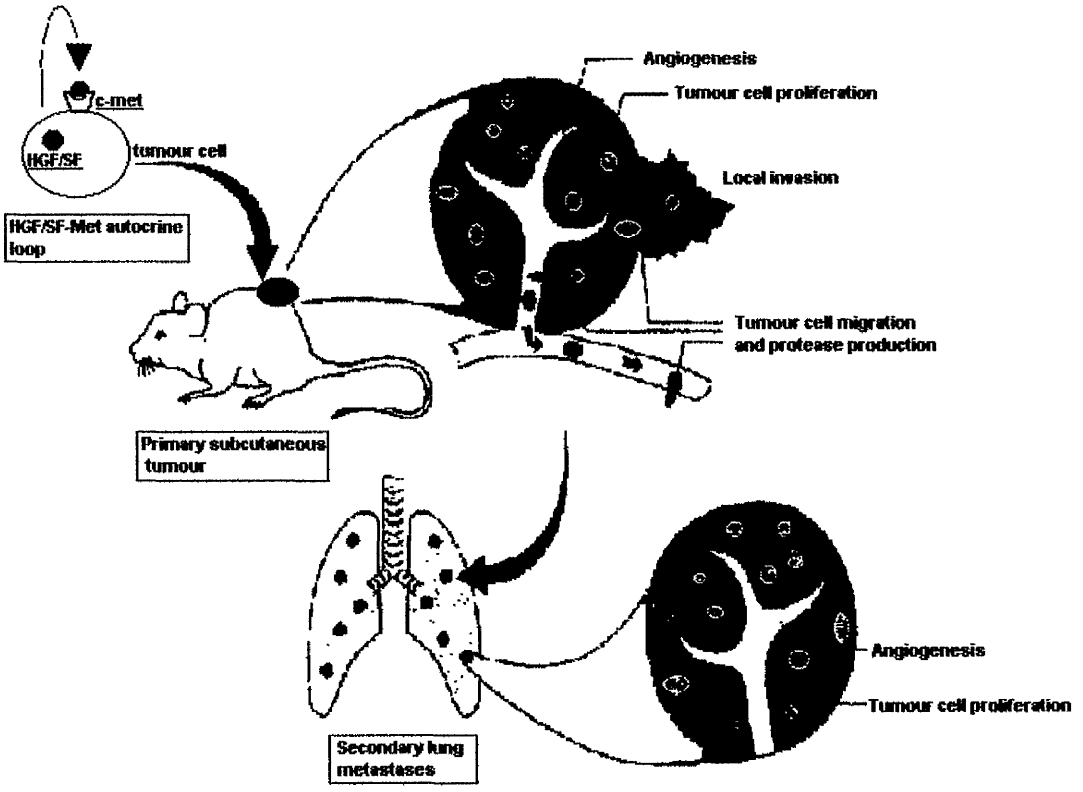


Figure 1.6

Illustration of the role of HGF/SF in tumorigenicity and invasion/metastasis. Autocrine HGF/SF-*c-met* loops in C127 and EMT6 breast cancer cell lines have been shown to induce metastases in distant organs when implanted into nude mice. It is thought that HGF/SF-*c-met* loop promotes tumour growth, invasion, motility and angiogenesis that are essential steps of disease progression.

shown to enhance *in vivo* metastatic ability [247]. These studies demonstrate that autocrine expression of HGF/SF and *c-met* strongly promote metastases. The mechanisms that contribute to this observation may include increased cell growth and angiogenesis as discussed above. In addition, HGF/SF stimulates cell dissociation and motility. Finally HGF/SF stimulates the production of proteases that may participate in tumour cell-mediated degradation of basement membrane and ECM.

HGF/SF, *c-met* and their relationship with tumorigenesis

HGF/SF and *c-met* have been implicated as mediators of tumour metastatic progression *in vivo* because of their profound stimulatory effects on tumour cell functions that are central to the process of metastasis. The HGF/SF- *c-met* system has been shown to induce tumour formation either through the oncogene tpr-met or by autocrine signalling [116]. *C-met* was first identified as a proto-oncogene through its ability to transform NIH 3T3 cells in the rearranged oncogene tpr-met [46,215]. Expression of this oncogene in transgenic mice has been associated with the development of mammary hyperplasia and tumours [152].

Autocrine expression of HGF/SF and *c-met* has been shown to induce transformation and tumorigenicity *in vitro*. NIH 3T3 cells naturally produce HGF/SF but little or no *c-met* protein. Transformation and tumorigenicity is induced by genetic transfer of *c-met* [239]. In addition, spontaneously transformed NIH 3T3 cells often overexpress *c-met* protein [40]. In this murine system, transfection of human *c-met* does not result in

transformation unless human HGF/SF is co-transfected [239]. This observation indicates that overexpression of unaltered *c-met* does not induce transformation of cells that do not produce or have access to HGF/SF. Similar observations have been reported in C127 breast cancer cells, SK-LMS-1 leiomyosarcoma cells and NBT-II bladder carcinoma cells [10,115].

The biological processes underlying HGF/SF-*c-met* induced tumorigenicity are not fully understood. However, three mechanisms may play a role in this process. Firstly, HGF/SF-*c-met* is mitogenic to a number of cells and uncontrolled autocrine induced cell growth contributes to tumour formation. In support of this hypothesis tumour formation in HGF/SF-*c-met* stimulated NIH 3T3, C127 and SK-LMS-1 cells involves increased cell growth [115]. Secondly, HGF/SF-*c-met* has been shown to induce angiogenesis and this may contribute to tumour formation. Finally, HGF/SF induces the expression of both uPA and its receptor which are necessary for HGF/SF activation [114].

HGF/SF and *c-met* in human cancers

The expression of HGF/SF and its receptor has been investigated in numerous human cancers. The following changes have been observed in tumour: overexpression, aberrant expression, receptor mutation and possible over-autophosphorylation [122]. The *c-met* proto-oncogene is overexpressed in several types of human cancer. Initial studies by Di Renzo *et al* and Prat *et al* reported overexpression of *c-met* in human carcinomas of the colon, stomach, pancreas, small bowel, thyroid, ovary, kidney and brain [54,229].

Gastro-intestinal cancers

Both *c-met* and HGF/SF overexpression have been reported in gastrointestinal cancers. High levels of tumour HGF/SF has been associated with reduced survival in patients with squamous cell carcinomas of the oesophagus [286]. In gastric cancers frequent amplification of the *c-met* gene has been observed [144]. Overexpression of *c-met* mRNA in human gastric cancer appears to be associated with local tumour invasion and lymph node metastasis [146]. Circulating levels of HGF/SF are increased in patients with primary and recurrent gastric cancers. Increased levels have been found to correlate with histological tumour and venous invasion [293]. *C-met* mRNA levels are increased in both colonic adenomas and colorectal cancers [155]. In a series of 123 colon cancers, Flavia *et al* observed that *c-met* was overexpressed in 50% of primary tumours and in 70% of metastases, suggesting a link between overexpression and disease progression [71]. Tumour concentration of HGF/SF in colon cancers is increased when compared to normal tissue [74]. Furthermore, serum concentration of HGF/SF have been shown to correlate with Dukes classification, lymph node involvement and liver metastases [74]. Co-expression of HGF/SF and *c-met* have been observed in human pancreatic cancers and levels of mRNA were significantly increased in tumour when compared to normal tissue [56,63]. In hepatocellular carcinomas *c-met* expression was found to correlate with reduced tumour differentiation [284].

Urinary tract tumours

In human prostate cancers *c-met* expression is increased when compared to normal tissue. Increased *c-met* expression also correlated to higher tumour grade [223]. Co-expression of both *c-met* and HGF/SF is reported in human bladder cancers [129]. In addition, elevated levels of HGF/SF are associated with high grade tumours in human bladder cancer [129].

Thyroid cancer

C-met overexpression has been detected in thyroid cancers. The significance of overexpression is the subject of controversy. One study demonstrated a relationship between overexpression and poor prognostic histology [55]. However, Belfiore *et al* demonstrated a relationship between low levels of expression and distant metastases [9]. Another study demonstrated *c-met* expression in the majority of papillary carcinomas with a relationship to poorly differentiated tumours [253].

Lung cancer

Levels of immunoreactive HGF/SF are significantly higher in non-small cell carcinomas when compared to other tumour types [266]. The level of tumour HGF/SF was found to be a good indicator of patient survival. Other studies have shown a relationship between *c-met* expression and prognosis in lung cancer [289]. Although HGF/SF levels are

elevated in the sera of lung cancer patients, no relationship between serum levels and prognosis have been reported [291]. HGF/SF is present in a large proportion of malignant pleural effusions due to secondary spread from a number of tumour types [62,134].

Brain and tumours of nervous system

Gliomas have been found to express both HGF/SF and *c-met*. In addition gliomas have been observed to secrete active HGF/SF. In peripheral nerve sheath tumours, expression of HGF/SF and *c-met* is negative in benign and positive in malignant tumours [234]. HGF/SF and *c-met* expression has also been found to correlate with grade of malignancy [143].

Other tumours

C-met overexpression and co-expression of both HGF/SF and *c-met* has been observed in several osteosarcoma cell lines [69]. Skin cancers and haemopoetic malignancies also demonstrate aberrant HGF/SF and *c-met* expression [123].

Proposal for thesis

HGF/SF appears to function as a paracrine regulator for the growth, dissociation, motility and invasiveness of epithelial cells and for angiogenesis. In addition HGF/SF-*c-met* may be an important stromal-epithelial regulator of epithelial morphogenesis in development. However, aberrant HGF/SF-*c-met* signalling has been shown to induce transformation and promote tumour invasion and metastasis both *in vitro* and *in vivo* (Figure 1.7) [115].

HGF/SF was first identified as a soluble factor that dissociates human breast epithelial cells in culture [279]. HGF/SF-*c-met* may also play a role in morphogenesis of breast ducts. Human breast epithelial cells form lumen-like structures in the presence of HGF/SF *in vitro* [303]. HGF/SF-*c-met* is therefore a candidate gene participating in stromal-epithelial regulation of breast duct development. This is supported by evidence that breast epithelial cells *in vitro* can regulate fibroblast production of HGF/SF [267,268]. Furthermore both HGF/SF and *c-met* expression are induced by oestrogen [157,186]. Loss of regulation of morphogenic pathways may result in uncontrolled cell dissociation, motility, invasiveness and ultimately metastasis. There is evidence that aberrant HGF/SF-*c-met* expression may occur in human breast cancer. Levels of HGF/SF are increased in breast cancer homogenates [331]. Furthermore, LOH of *c-met* in human breast cancer is associated with both reduced disease-free and overall survival [16]. The expression status of *c-met* in breast is controversial. Initial studies failed to detect *c-met* expression in breast-tissue, although it was known that breast epithelial cells responded

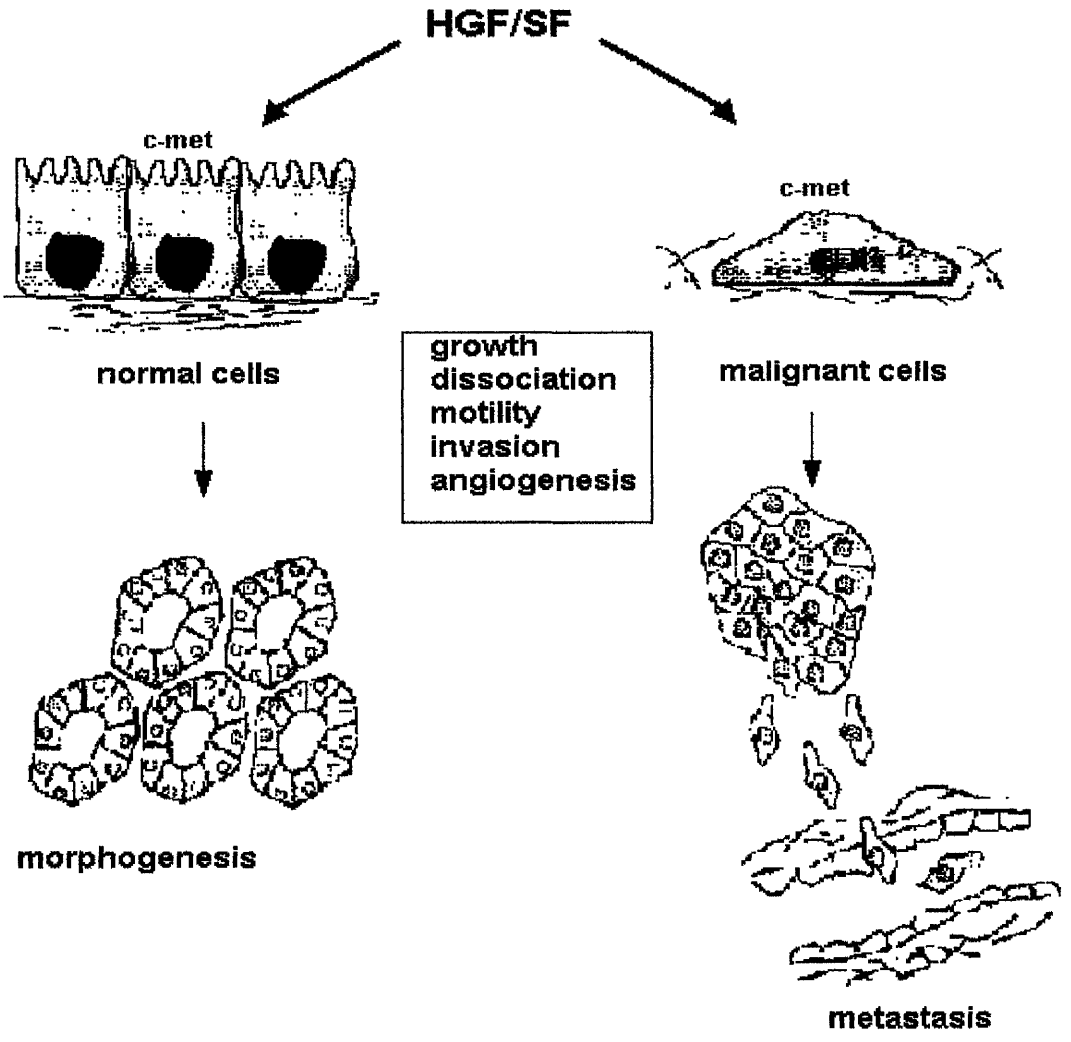


Figure 1.7

Hypothetical illustration of HGF/SF-*c-met* interaction in normal and malignant cells. In normal cells, HGF/SF promotes co-ordinated cell growth, dissociation, invasion and motility which manifest as morphogenesis. In malignant cells these component actions are excessive and exhibit loss of co-ordination resulting in metastasis. Functional E-cadherin is an important determinant in HGF/SF induced morphogenesis and metastasis.

to HGF/SF [54,229]. However, Tsarfaty *et al* detected *c-met* expression in breast cancers and in adjacent tumour-free breast tissue [303]. The aim of this thesis is to further investigate the role of HGF/SF and *c-met* in human breast cancer.

Aims of study

1. To investigate the frequency and significance of LOH of *c-met* in patients with breast cancer.

2. To investigate HGF/SF and *c-met* expression in patients with breast cancer.

3. To correlate HGF/SF expression with tumour proliferation and angiogenesis in patients with breast cancer.

Chapter 2: Investigation on frequency of loss of heterozygosity of the *c-met* proto-oncogene in human breast cancer

Introduction

In 1992 Bieche *et al* detected a high frequency of allelic loss (40.5%) using the pmet H polymorphic probe among 121 informative (heterozygous) patients at the *c-met* locus (7q31) with primary breast cancer [16]. In addition, these patients demonstrated significantly shorter metastasis-free and overall survival. This study provided the first *in vivo* evidence of a link between abnormal HGF/SF-*c-met* function and disease progression in breast cancer. Bieche *et al* concluded that an important tumour or metastasis suppressor gene was present at the *c-met* locus. This observation had not been reported before and required further investigation.

Genetic events resulting in the development of cancers are thought to involve activation of cellular oncogenes and loss of function of tumour suppressor genes. These events are thought to occur in a multistep fashion. Loss of heterozygosity (LOH) analysis of polymorphic DNA markers point to chromosomes and subregions of chromosome arms likely to harbour tumour suppressor genes [51]. However only in a few instances have actual genes, such as *Rb* or p53, relevant to allelic loss been identified. Breast cancers appear unusual among human tumours for the great degree of genetic heterogeneity displayed. LOH in carcinoma of the breast affects at least 13 chromosome arms. According to Knudson's Hypothesis this would suggest that more than 20 genetic loci

may undergo mutation followed by allelic loss during the initiation and progression of breast cancer [140]. Alternatively, many of these LOH could be irrelevant and may result from chromosome disintegration. This is in contrast to colorectal cancer where allelic loss is mainly restricted to six chromosomes [68].

LOH studies from many groups have shown that the following regions of the genome undergo allelic loss at the approximate frequencies indicated (only frequencies of 30% or greater listed): Chromosome 1p (40%), 3p (40%), 6q (35%), 8p21-22 (55%), 9p (65%, attributed to the CDK inhibitor p16), 11q (40%), 13q12-13 (35%, potentially related to BRCA-2), 16q(50%), 17p (55%, attributed in part to p53), 17q (40%), 18q (35%), 22q (40%) [27,28,30,32,38,51,154,191,336,342]. A recent genome-wide search for LOH in 56 regions of the genome in human primary breast cancer confirmed a variable pattern of allelic loss in the majority of cases with the most frequently deleted regions being 8p, 11q, 16q and 17p [135]. The possible involvement of specific tumour suppressor genes in the later stages human cancer is not clear. Putative metastasis-related genes have been identified using cell lines and rodent models. These studies have linked metastatic phenotypes to genes such as NME-1 (17q), CDH1 (16q), TSG101(11p) and KISS1 (1q). In breast cancer relapse samples candidate metastasis related genes targeted by LOH have been identified on 3p21.3 and 16q22.2-23.2 [59].

Materials and methods

The use of restriction fragment length polymorphism (RFLP) analysis of DNA is a well established method of detecting tumour suppressor genes in human cancer. The pmet H probe recognises RFLP of 4.0 and 7.5 kb on Southern blot electrophoresis enabling heterozygous (informative) cases to be identified from blood analysis. Tumour DNA from informative cases are then analysed for LOH.

Preparation of probe

Identification of *c-met* locus in both blood and tumour DNA was performed using the pmet H probe obtained from American Type Culture Collection, Maryland, USA (ATCC). The pmet H probe is a 1.6kb fragment prepared from the plasmid pBR322. This probe recognises the 7.5 and 4.0 kb Taq1 restriction fragments. E.coli DH1 containing the plasmid pBR322 with the 1.6kb metH insert was rehydrated and grown overnight in a shaking incubator at 37^0C in 10ml of Luria Bertani medium (10gms bacto-tryptone, 5gms bacto-yeast extract, 10gms sodium chloride [NaCl] , 950mls distilled water) containing ampicillin at a final concentration of 0.1μg/ml. The culture was spun down at 300 rpm for 15 minutes. The cell pellet was retrieved, resuspended in 350μl of cold STET buffer (50mM Tris pH 8.0, 50mM EDTA, 8% sucrose, 5% Triton X-100) and 25μl of fresh cold lysosyme (10mg/ml) added. This was left on ice for 5 minutes, placed in boiling water for 60 seconds and then centrifuged at 13,000 rpm for 15 minutes. The supernatant was retrieved and 1/10 volume of 3M sodium acetate and

phenol equilibrated with 0.5M Tris (pH 8.0) added. The aqueous phase was then retrieved and plasmid DNA precipitated with 0.6 volume of propan-2-ol for 30 minutes at -20^{0}C. The precipitate was centrifuged at 13,000 rpm for 5 minutes. The plasmid DNA pellet was retrieved, washed in 95% ethanol, vacuum dried and dissolved in 100µl of water. Plasmid DNA was digested with EcoRI and SalI restriction enzymes (50µl plasmid DNA, 6.5µl KGB buffer [2x KGB: 200mM potassium glutamate, 50mM Tris-acetate, 20mM magnesium acetate, 100µg/ml bovine serum albumin, 1mM mercaptoethanol], 2.5µl EcoRI, 2.5µl SalI, 3µl spermidine) overnight at 37^{0}C. 4µl of loading mix (8gms sucrose, 2.4mls 50x electrophoresis buffer, 50mgs bromophenol blue) was added to the digest. A 1.0% low melting point agarose gel was prepared using electrophoresis buffer (50x: 2420gms Tris, 571mls glacial acetic acid, 1L 0.5M EDTA pH8, made up to 10L) and stained with ethidium bromide. The digests were electrophoresed next to a 1kb ladder at 40mAmp for three hours. Two bands of just greater than 4 and 1kb were identified corresponding to restriction digests analysed on agarose gels giving the EcoRI/SalI 4.2 and 1.25 kb fragments (Figure 2.1). The 1.2kb band was cut out, mixed with three times its weight of water and placed in a water bath for 10 minutes at 100^{0}C. This was divided into aliquots of 25µl and frozen at -20^{0}C until required.

Collection of paired samples of blood and tumour from primary breast cancer patients

Paired samples of tumour and blood from 111 patients treated with primary breast carcinoma at the Western Infirmary, Glasgow were studied. None of these patients had

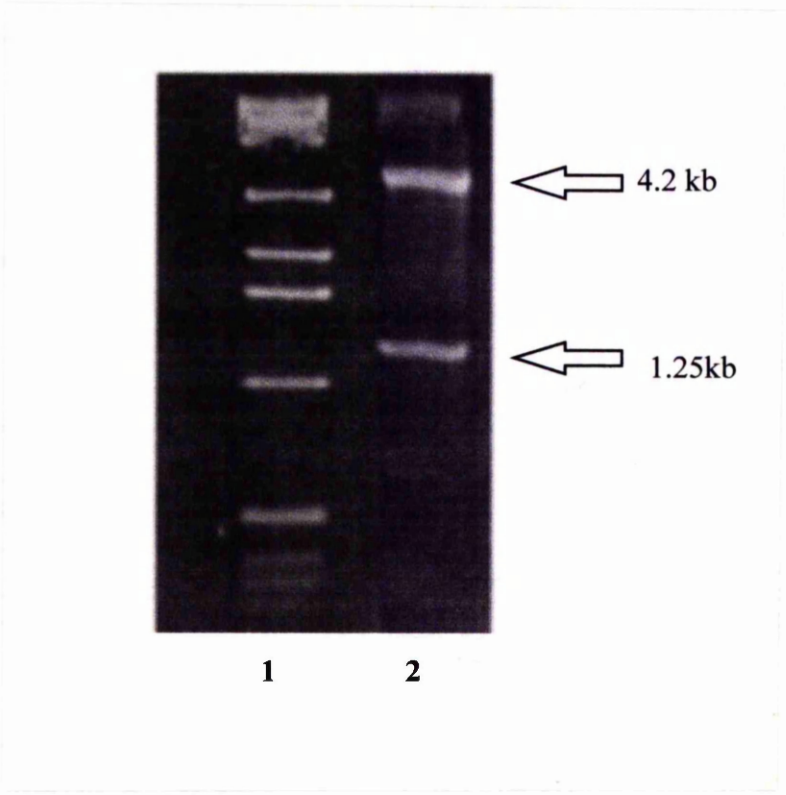


Figure 2.1

DNA electrophoresis of restriction digests with SalI and EcoRI of plasmid DNA.
Lane 1: 1kb ladder, lane 2: plasmid DNA digests.
4.2 and 1.25 kb fragments were detected.

received preoperative cytotoxic, endocrine or radiotherapy. Tumour and blood samples were collected and immediately frozen at -70^{0}C until needed. These samples were collected from a larger tumour bank for which patient consent and ethical committee approval had been obtained. The presence of tumour in samples investigated were confirmed by routine histopathology. Clinico-pathological data including age, tumour type, tumour size, histological grade, oestrogen receptor and axillary nodal status were retrieved.

De-oxyribonucleic acid (DNA) extraction from blood and tumour

Extraction of blood DNA

Two volumes of lysis buffer (10mM Tris pH 8.0, 0.1M EDTA pH 8.0, 20μg/ml pancreatic RNase, 0.5% sodium dodecyl sulphate [SDS]) was mixed in 5mls blood and then centrifuged at 5^{0}C for 15 minutes at 2500 rpm. The supernatant was then discarded and 2mls nucleic acid lysis buffer (40mls 5M NaCl, 5mls 1M Tris, 2mls 0.5M EDTA pH 8.2, 500mls distilled H_20), 175μl 10% SDS and 100μl proteinase K added. The mixture was incubated overnight at 37^{0}C in a water bath. The following morning, 700μl of 5M NaCl was added and the mixture centrifuged at 5^{0}C for 15 minutes at 2500 rpm. The supernatant was decanted and 700μl of phenol/chloroform (1gm hydroxyquinolone, 4gms Tris, 1.5L water saturated phenol made up to 1:1 mixture with chloroform) added. This was gently mixed and then centrifuged at 1200 rpm for 10 minutes at room temperature. The supernatant was decanted and an equal volume of ethanol added. The precipitated DNA was spooled onto glass rods, air dried and dissolved in 0.5ml of Tris-

EDTA (TE) buffer. Samples were stored at -20^{0}C until needed.

Extraction of tumour DNA

Breast tumour samples were thawed over ice and mechanically disaggregated in 200µl of 10% SDS and 2mls of nucleic acid lysis solution. This was placed in a universal container where 200µl of 10% SDS, 2mls of nucleic acid lysis solution and 200µl of proteinase K added. The mixture was incubated overnight at 37^{0}C. Afterwards, 1.4mls of 6M NaCl was added and centrifuged at 2500 rpm for 15 minutes at 15^{0}C. The supernatant was retrieved and 700µl of phenol/chloroform added, mixed and centrifuged at 1000 rpm for 10 minutes at room temperature. The DNA was precipitated by adding 3 volumes of ethanol. The DNA was spooled onto glass rods, air dried for 10 minutes, dissolved in 0.5ml TE buffer and stored at -20^{0}C until needed.

DNA quantitation

DNA was quantified by spectrophotometry. 10µl of DNA solution was diluted in 990 µl of TE buffer and mixed thoroughly. The optical density (OD) was then measured at 260nm. DNA concentration was then calculated as the product of OD260 x dilution factor (100) x 50.

Genomic DNA digestion

Blood DNA was first analysed to determine informative (heterozygous) cases for the *c-met* gene. Tumour samples from these cases were then analysed for LOH. The protocols for genomic digestion, electrophoresis, preparation of filters, hybridisation and autoradiography were the same for both instances. Restriction digests of 40µl total volume were prepared as follows; 5µg sample DNA, 4µl buffer (react 2 for Taq 1, Gibco BRL, Paisley, Scotland), 3µl Taq 1, 2µl spermidine, variable volume of deionised water depending on DNA concentration. The digests were incubated in a waterbath set at 65^0C overnight.

Southern blot electrophoresis

Preparation of gels

DNA digests were separated by electrophoresis in 0.7% agarose gels. This was prepared by dissolving 2.8mgs of agarose in 400mls of electrophoretic buffer. This was boiled in a microwave oven for 5 minutes and stained with 12µl of ethidium bromide. A 22 lane plate was prepared and gel poured in when below 60^0C. When set the gel was submerged in a sufficient volume of electrophoretic buffer. The DNA digests were stained with 4µl of bromophenol blue and 35µl of DNA digest loaded into the wells. Electrophoresis was commenced at 240mA for three hours. The gel was retrieved and treated with 0.25M hydrochloric acid for 15 minutes followed by 30 minutes of denaturation solution (1.5M NaCl, 0.5N sodium hydroxide) and a further 30 minutes

with neutralisation solution (1M Tris pH 7.4, 1.5M NaCl).

Southern blot transfer

Separated DNA fragments were transferred to a Hybond membrane according to the method described by Southern (Figure 2.2) [277]. This was left at room temperature for 2 days. The membrane was retrieved and DNA fixed by baking at 80^0C for 5 hours.

Membrane treatment

Prehybridisation

Membranes were rolled into a Hybaid bottle to which 7mls of hybridisation solution (6x saline-sodium citrate [SSC], 0.5% SDS, 5x Denhardt's solution) and 70µl of salmon sperm DNA (denatured at 100^0C for 10 minutes) was added. The bottles were incubated in a Hybaid oven at 65^0C overnight.

DNA radiolabelling

The pmet H probe was radiolabelled using a random primer labeling kit (Roche Diagnostics Ltd. [Boerhinger Mannheim] East Sussex, UK). 25µl of the pmet H probe was boiled for 10 minutes and then incubated for 5 minutes at 37^0C. To this was added 6µl of deoxyadenosine triphosphate, deoxyguanosine triphosphate and thymidine triphosphate mixture, 4µl reaction mixture, 1.5µl Klenow enzyme and 5µl of

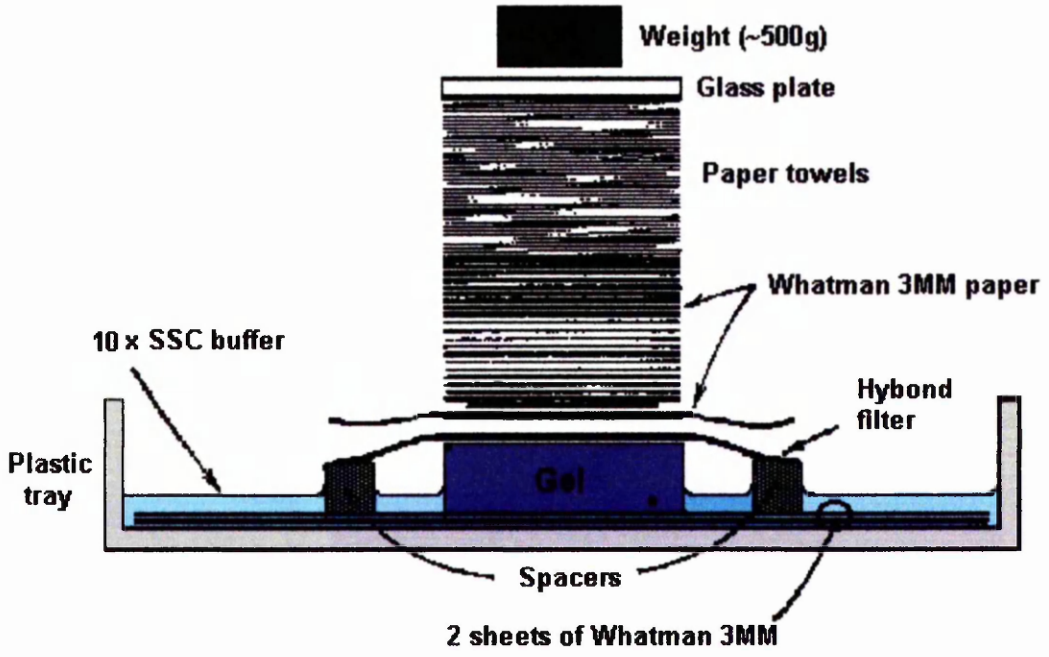


Figure 2.2

Diagram of apparatus used for Southern blot of DNA onto Hybond membrane.

radiolabelled α^{32}p deoxycytidine triphosphate. The mixture was incubated at 37^0C for two hours and labelled probe separated with a Sephadex G50 column washed down with 1x SSC/0.1% SDS. Approximately 0.5ml of the peak radioactive solution was collected for probing.

Hybridisation of filters.

The labelled probe was denatured by boiling for 10 minutes and mixed into the hybridisation solution. The membrane was hybridised at 65^0C in a Hybaid oven overnight.

Washing of membrane

Excess radioactive probe was washed from the hybridised membrane using 2xSSC/0.1% SDS at 65^0C in Hybaid oven for 30 minutes. This was repeated again in a water bath of 2x SSC/0.1% SDS until the radioactive background was less than 10 counts per second. The filter was blotted dry between sheets of 3mm filter paper and then protected with a polythene sheet.

Autoradiography

The membrane was placed in a light-proof cassette containing intensifying screens with Kodak Diagnostic X-OMAT AR5 fast film. The cassette was left overnight at -20^{0}C and the film developed using an AGFA X-ray film processor (Curix 60 process). If the hybridisation signals were not strong enough the filter was exposed for a longer time. If the background was intense the membrane was washed and then re-exposed.

Results

The pmet H probe

EcoRI/SalI digests of pBR322 plasmid with 1.6kb *c-met* insert produced the expected fragments of 4.2 and 1.25 kb on agarose electrophoresis (Figure 2.1).

Patient population

111 patients with primary breast cancer were investigated for LOH of *c-met* in this study. The clinico-pathological variables of this group are summarised (Table 2.1).

Blood and tumour DNA analysis

The pmet H probe detected Taq 1 RFLP of 7.5 and 4.0 kb (Figure 2.3). Visual inspection of ethidium bromide-stained gels and hybridisation signal intensity were used to compare the amount of DNA loaded in the different lanes. The intensity of hybridisation to each restriction fragment was visually assessed. Samples with low levels of DNA loading were re-evaluated. Analysis of blood DNA demonstrated that 52/111 patients (47%) were heterozygous for the Taq 1 RFLP of 7.5 and 4.0 kb. 36/111 cases (32%) were homozygous for the 7.5kb allele and 23/111 cases (21%) homozygous for the 4.0kb allele (Figure 2.3). In this study, of the 52 heterozygous

Table 2.1

Clinico-pathological variables of breast cancer patients.

Clinico-pathological variable	Number of Patients
Age	Median 60 yrs (range 27-89 yrs)
Tumour size	Median 25 mm (range 7-90mm)
<30mm	85
≥ 30mm	26
Tumour grade	
1	16
2	65
3	30
Lymph node status*	
Negative	55
Positive	55
Oestrogen receptor status**	
Negative	40
Positive	60
Histological Type	
Ductal	107
Lobular	4

* Lymph node status not known in one patient

** Oestrogen receptor status not known in 11 patients

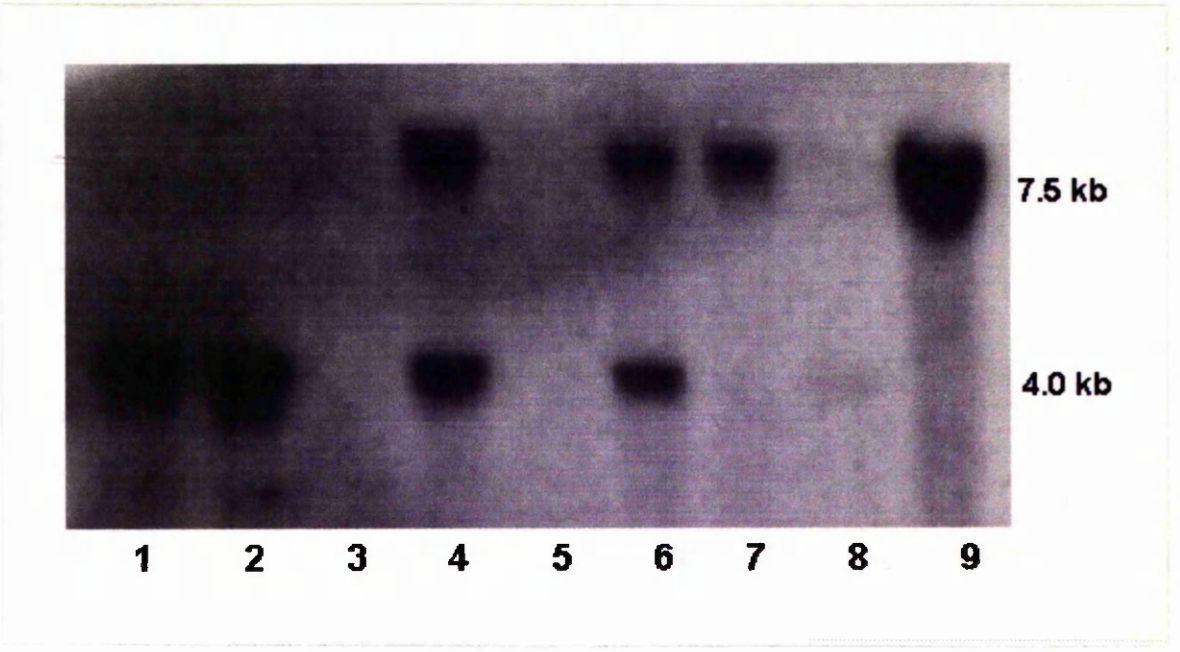


Figure 2.3

Autoradiographs of blood DNA demonstrating RFLP of 7.5 and 4.0 kb. Lanes 1, 2, 7 and 9 are homozygous for either 7.5 or 4.0 kb allele and are therefore non-informative. Lanes 4 and 6 are heterozygous. Lanes 3, 5 and 8 are blank.

cases, total LOH was detected in only 2 tumour samples, a frequency of 4% (Figure 2.4). Reduced signal intensity for the 7.5kb allele was observed in a further 5 cases. In this study therefore partial/total LOH was observed in 7/52 cases, a frequency of 13%.

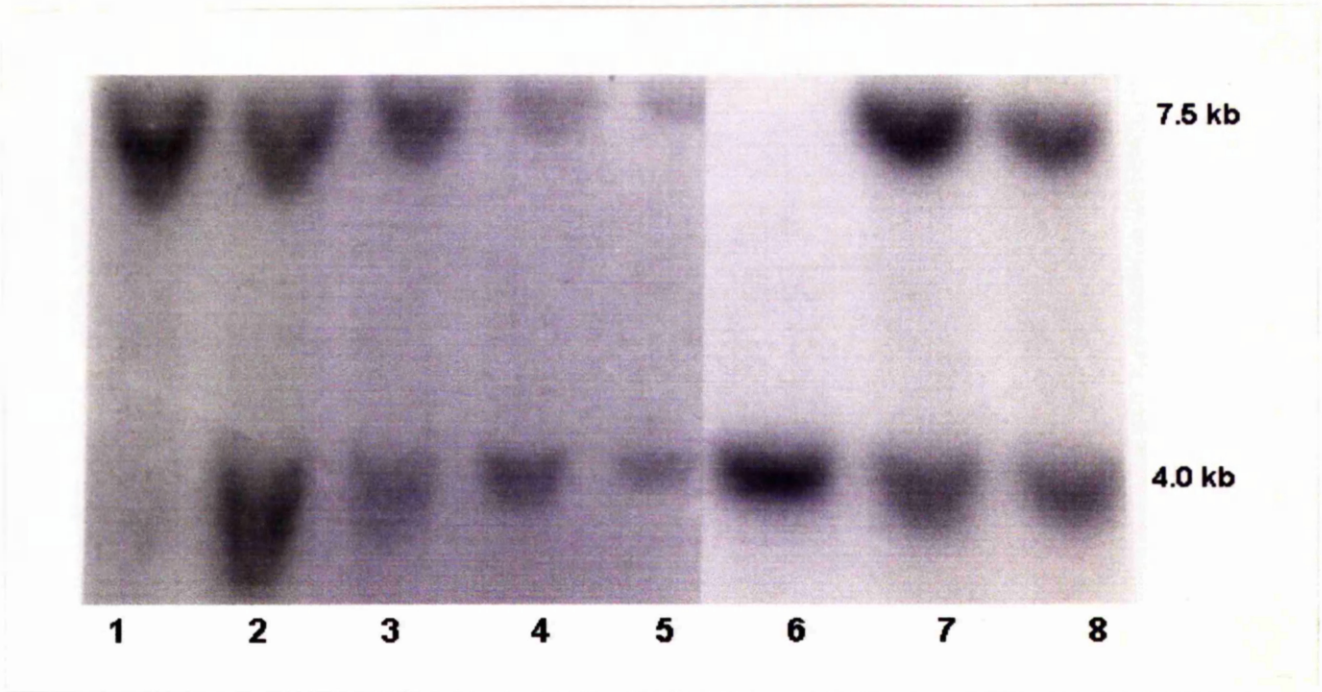


Figure 2.4

Autoradiographs of breast cancer DNA demonstrating RFLP of 7.5 and 4.0 kb in patients found to be heterozygous for *c-met* following blood DNA analysis. LOH was detected in only two patients (lanes 1 and 6).

Discussion

Functional inactivation of a tumour suppressor gene often involves deletion of the normal allele to unmask the mutated allele. Chromosomal regions with frequent deletions are thought to harbour putative tumour suppressors [33]. Frequent LOH at a given chromosomal region has been interpreted as evidence that the affected region may contain a tumour-suppressor gene that is inactivated during the malignant process [140]. Chromosome arms demonstrating LOH of greater than 20% are thought to be of potential interest in breast cancer [135]. In a review of published allelotype data from over 30 studies comprising more than 1000 breast tumour specimens, Devilee *et al* identified 11 chromosome arms showing LOH in greater than 25% of investigated tumours and 2 (16q and 17p) with LOH greater than 50% [51].

Cytogenetic studies have revealed frequent chromosome 7 abnormalities in several tumour types including breast, gastric and prostate cancers [92,287,315,330]. In breast cancers several karyotypes with numerical alterations (polysomy or monosomy 7 and 7p) and structural abnormalities on both arms of chromosome 7 have been reported [17]. Furthermore, *in vitro* studies have also suggested the presence of a tumour suppressor gene on chromosome 7. Microcell transfer of human chromosome 7 into a murine squamous carcinoma cells has been shown to inhibit tumorigenicity [343]. Similarly, insertion of intact human chromosome 7 into an immortalised human fibroblast cell line with 7q31-32 LOH suppressed the immortality of the cells and restored the ability for senescence [212]. However, cytogenetic methods detect only gross chromosomal changes.

LOH analysis with either RFLP/microsatellite markers is a sensitive molecular method to screen for changes involving allele loss and by presumption, markers of tumour suppressor gene loss in tumours. Several investigators have suggested the presence of an important tumour suppressor gene on 7q31.1 in a variety of human primary cancers (Table 2.2). Some of these studies have also shown that LOH at 7q31.1 correlated with poor prognosis suggesting a role in tumour progression. LOH at 7q31.1 has been linked to tumour aggressiveness and poor prognosis in several human cancers including gastric, prostate, thyroid, ovarian and breast cancer suggesting a possible role in the development or progression of these malignancies [16,137,145,288,347].

Microsatellite marker studies have attempted to locate the precise position of the smallest common deleted region of 7q31.1 in a variety of cancers which may identify the putative tumour suppressor gene (Figure 2.5). Zenklusen *et al* have used several polymorphic microsatellite markers in an attempt to determine the location of the putative tumour suppressor gene on chromosome 7q in carcinomas of the breast, prostate, head and neck, colon and ovary [342,344,345,346]. These studies have shown that the smallest commonly deleted region is located at D7S522. Other investigators, studying prostate tumours, suggested, that the putative tumour suppressor gene could be located around D7S480 and D7S486 [151,288]. Similarly in gastric and thyroid cancers the smallest commonly deleted region was located around D7S480 [209,347].

Several studies have attempted to identify the gene present in the smallest commonly deleted region of 7q31.1. Early studies had suggested that *c-met* may have been the

Table 2.2

Table of 7q31 LOH studies in human cancers.

Tumour type	Patient number	LOH frequency	Reference
Breast	231	40.5%	Bieche *et al* 1992 [16]
Breast	31	83%	Zenklusen *et al* 1994 [342]
Breast	73	12.5%	Lin *et al* 1996 [153]
Breast	113	44%	Bieche *et al* 1997 [17]
Breast	683	19%	Devilee *et al* 1997 [52]
Breast	49	20%	Driouch *et al* 1998 [59]
Prostate	16	83%	Zenklusen *et al* 1994 [345]
Gastric	53	34%	Nishizuka *et al* 1997 [209]
Colon	8	80%	Zenklusen *et al* 1995 [344]
Thyroid	42	48%	Zhang *et al* 1998 [347]
Ovarian	22	50%	Kioke *et al* 1997 [137]

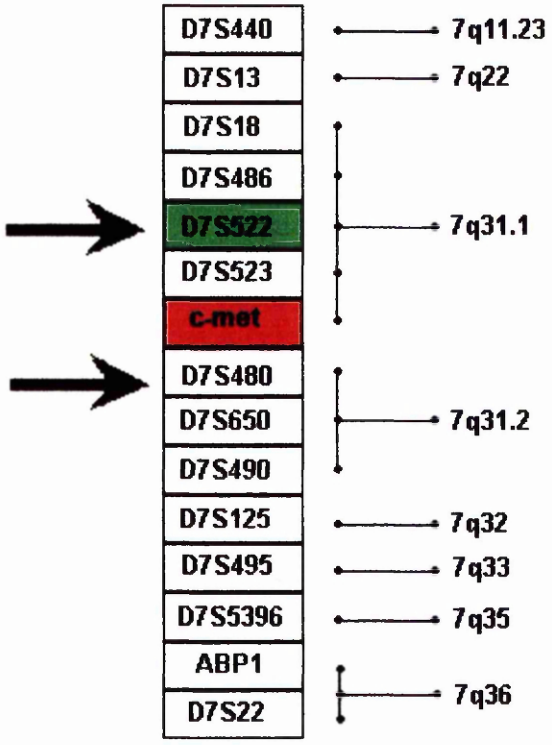


Figure 2.5

Microsatellite map of the long arm of chromosome 7. Arrows point to the smallest regions of frequent deletion as observed in human breast cancers. These are separate from the *c-met* locus at 7q31.1. DS7522 also harbours the fragile site FRA7G which may explain the wide variability in frequency of LOH observed at 7q31.1.

candidate gene. Deletion mapping of 7q31 in breast cancer has identified a 1000kb deletion including *c-met* but no other known genes [153]. Microsatellite marker experiments in breast cancer observed the smallest commonly deleted region of 7q31.1 to be at D7S522 [342]. However, the *c-met* locus is situated 500kb telomeric to D7S522 and is separate from the other frequently deleted regions D7S480 and D7S486 (Figure 2.5) [116]. In a recent series of 113 primary breast cancers, Bieche *et al* reported the smallest commonly deleted region of 7q31 lay between D7S480 and D7S650 which are telomeric to *c-met* [17]. Finally, mutation analysis of *c-met* in prostate cancers suggested the gene was rarely mutated in this disease [116]. These observations do not support *c-met* as the candidate tumour suppressor gene at 7q31.

The frequency and significance of LOH at 7q31 in human breast cancer has been the subject of several studies. In a series of 121 informative primary breast cancers, Bieche *et al* first reported a frequency of LOH at 7q31 of 40.5% [16]. Furthermore, breast cancer patients with LOH at 7q31 experienced a significant reduction in both disease-free and overall survival. The utilisation of polymorphic markers later located the common region of LOH to be distributed around the D7S522 probe and observed LOH in 83% of 31 cases [342]. Subsequent analysis using a combination of RFLP and microsatellite markers in another series of 113 primary breast cancers demonstrated LOH of a frequency of 46% around D7S522 [17]. Previous independent studies using similar probes (3 pmet H, one JCZ67 and one pmet H/XV-2c) had reported a frequency of LOH at 7q31 of less than 10% [41]. In a series of 73 breast cancers, Lin *et al* observed LOH at 7q31 in 12.3% of informative cases [153]. In addition, a recent genome wide study of 115

primary breast cancers, looking for LOH patterns using 184 microsatellite markers, detected allelic loss at 7q31 at a frequency of just below 20% [135]. Finally, a multicentre study of 683 breast cancers, using three polymorphic markers for 7q31-32, detected an average LOH of 19% [52]. Disease-free and overall survival of the patients whose tumours carried LOH at 7q31 did not differ significantly from those that did not. This study, the largest to date, concluded that LOH at 7q31 was neither an important genetic alteration nor a strong determinant of disease outcome in breast cancer. Recently members of the Bieche group reported LOH at 7q31 at a frequency of less than 20% in breast cancer patients [59]. They concluded that the involvement of 7q31 was controversial in breast cancer.

Several reasons can be forwarded to explain the wide range of LOH at 7q31 reported by various investigators (Table 2.2). Tumour heterogeneity and inclusion of non-malignant tissue may obscure allelic losses. Small sample numbers and differences in patient selection may be important. Observer variation may play a part. In a double blind analysis of a subset of the 683 breast cancers investigated for allelic loss at 7q31, Devilee *et al* observed variation in LOH scoring of 12% [52]. Recently the presence of a chromosomal fragile site, FRA7G, has been located to D7S522 at 7q31.1 [109]. Fluorescent in-situ hybridisation analysis of human prostate cancers demonstrated FRA7G breakage throughout the D7S522 region. The presence of a fragile site within 7q31.1 suggests deletions in this region may be the result of the intrinsic instability rather than the presence of a tumour suppressor gene [116].

The results of the present study do not support the presence of a tumour suppressor gene at 7q31.1, specifically the *c-met* locus, in human breast cancer. This observation has now been subsequently confirmed by several recent independent studies [52,59,135,153]. It is possible that the wide range of LOH observed at 7q31.1 is partly the function of a naturally occurring chromosomal fragile site present at D7S522.

Chapter 3: HGF/SF-*c-met* ligand receptor system in human breast cancer

Introduction

HGF/SF is a mesenchymal derived growth factor that is active through a receptor encoded by *c-met* in a wide variety of epithelial cell types and endothelial cells. Through its receptor HGF/SF promotes not only cell growth but also cell dissociation, motility, invasiveness and angiogenesis. This biological profile has led to the hypothesis that aberrant function of the HGF/SF-*c-met* ligand receptor system may play a role in tumour progression in human cancers [138]. In support of this hypothesis, aberrant HGF/SF-*c-met* signalling has been shown to promote both tumour invasiveness and metastasis *in vitro* and *in vivo* [115].

Benign and malignant human breast epithelial cells in culture demonstrate dissociation and increased motility in response to HGF/SF [26,280]. This response to HGF/SF is related to the degree of differentiation, well-differentiated cells demonstrating less motility when compared to poorly differentiated cells. Breast epithelial cells form lumen-like structures *in vitro* in response to HGF/SF, suggesting a possible role in morphogenesis [303]. These studies indicate that HGF/SF-*c-met* may be involved in both physiological and pathological processes occurring in breast. HGF/SF expression has been detected by immunohistochemistry in breast tissue [328]. Furthermore, HGF/SF levels are significantly higher in breast cancer homogenates when compared to levels detected in normal breast tissue [331].

The expression status of *c-met* in breast epithelium has been the subject of controversy. Prat *et al* detected immunoreactive *c-met* in 6/25 human primary breast cancers and 0/3 samples of normal breast tissue whereas Di Renzo *et al* detected *c-met* mRNA in 2/2 samples of normal breast and 0/15 samples of human breast cancer [54,229]. These studies based on small sample numbers require cautious interpretation. Tsarfaty *et al* detected epithelial immunoreactive *c-met* in all 50 samples taken from patients with primary breast cancer. However, expression of *c-met* was higher in adjacent tumour-free breast epithelial cells when compared to malignant epithelial cells [303].

To further investigate the HGF/SF-*c-met* ligand receptor system in human breast cancer, expression of both *c-met* protein and HGF/SF was determined in a series of benign and malignant breast tissues. Expression of these factors was then related to clinico-pathological variables, disease-free and overall survival by both univariate and multivariate analysis.

Materials and methods

Tissue samples

Samples of archival primary breast tumour, obtained between 1987-1989, with a median follow-up of 61 months, were retrieved for 73 patients that underwent definitive surgery for breast cancer at the Western Infirmary, Glasgow. None of these patients had received preoperative cytotoxic, endocrine or radiotherapy. These samples formed part of a tumour bank, for which patient consent and ethical committee approval had been obtained. This consisted of tissue samples packaged in aluminium foil, snap frozen and stored in liquid nitrogen. Clinico-pathological variables (age, axillary nodal status, tumour size, histological grade, oestrogen receptors and vascular invasion), disease-relapse and survival data were obtained from patient casenotes. In addition, 10 samples of tumour-free breast tissue were obtained (nine samples from patients undergoing mastectomy and one sample of breast tissue from a reduction mammoplasty). In the case of tissues obtained at mastectomy, samples were both macroscopically and histologically free from the presence of tumour.

Cell line

The HT29 colonic carcinoma cell line was used as a positive and quality control for the detection of *c-met* protein [88]. The cells were grown at 37^0C in RPMI 1640 medium supplemented with 10% foetal calf serum in a 5% CO_2-water saturated atmosphere until confluent.

Tissue preparation

HT29 colon carcinoma cell lines

The cells were washed twice with phosphate buffered saline (PBS)/EDTA and incubated with trypsin/EDTA for 10 minutes at room temperature. This was centrifuged at 1000 rpm for 5 minutes and the supernatant discarded. The cells were washed with PBS and centrifuged again at 1000 rpm for 5 minutes. The resultant cell pellet was dissolved in lysis buffer (2mM Tris (pH 7.5), 50mM NaCl, 0.5% (w/v) sodium deoxycholate, 1% (v/v) Nonindet P-40, 0.1% (w/v) SDS, 1mM phenylmethyl-sulphonyl fluoride, 1mg/ml aprotinin) [256]. This was centrifuged for 15 minutes at 12000g and the supernatant aliquoted and stored at -70^{0}C.

Breast tissue samples

Blocks measuring 5mm^3 were cut from the frozen tumour samples for homogenisation. The remaining sample was stored at -70^{0}C for further immunohistochemical studies. Haematoxylin and eosin (H&E) sections were also prepared from these samples to confirm presence of tumour. Tissues were ground into a powder using a pestle and mortar in liquid nitrogen. The powder was then dissolved in 0.25ml of lysis buffer. This was centrifuged for 15 minutes at 12000g and the supernatant aliquoted and stored at -70^{0}C.

Protein concentration

The protein concentrations of tissue homogenates were measured using the Pierce BCA protein assay kit (Pierce, Illinois, USA). This assay is based on the reaction of protein with Cu^{2+} to yield Cu^{+} which forms a purple coloured complex with bichinchoninic acid. This reaction is protein concentration dependent allowing spectrophotometric quantitation at 562nm. Protein standards of known concentration (200μg/ml-1200μg/ml) were prepared by dilution of bovine serum albumin (BSA). To 0.1ml of either standard or unknown protein, 2.0mls of Working Reagent was added and the mixture incubated for 2 hours at room temperature. Absorbance was measured at 562nm against a water blank. A standard curve was then plotted for the absorbance of protein standards and protein concentration of samples determined from this.

Discontinuous SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was conducted using a MINI-PROTEAN II Dual slab gel apparatus (Bio-Rad, Hertfordshire, UK, Figure 3.1). Protein electrophoresis was conducted using a 7.5% gel for *c-met* and a 12% gel for HGF/SF.

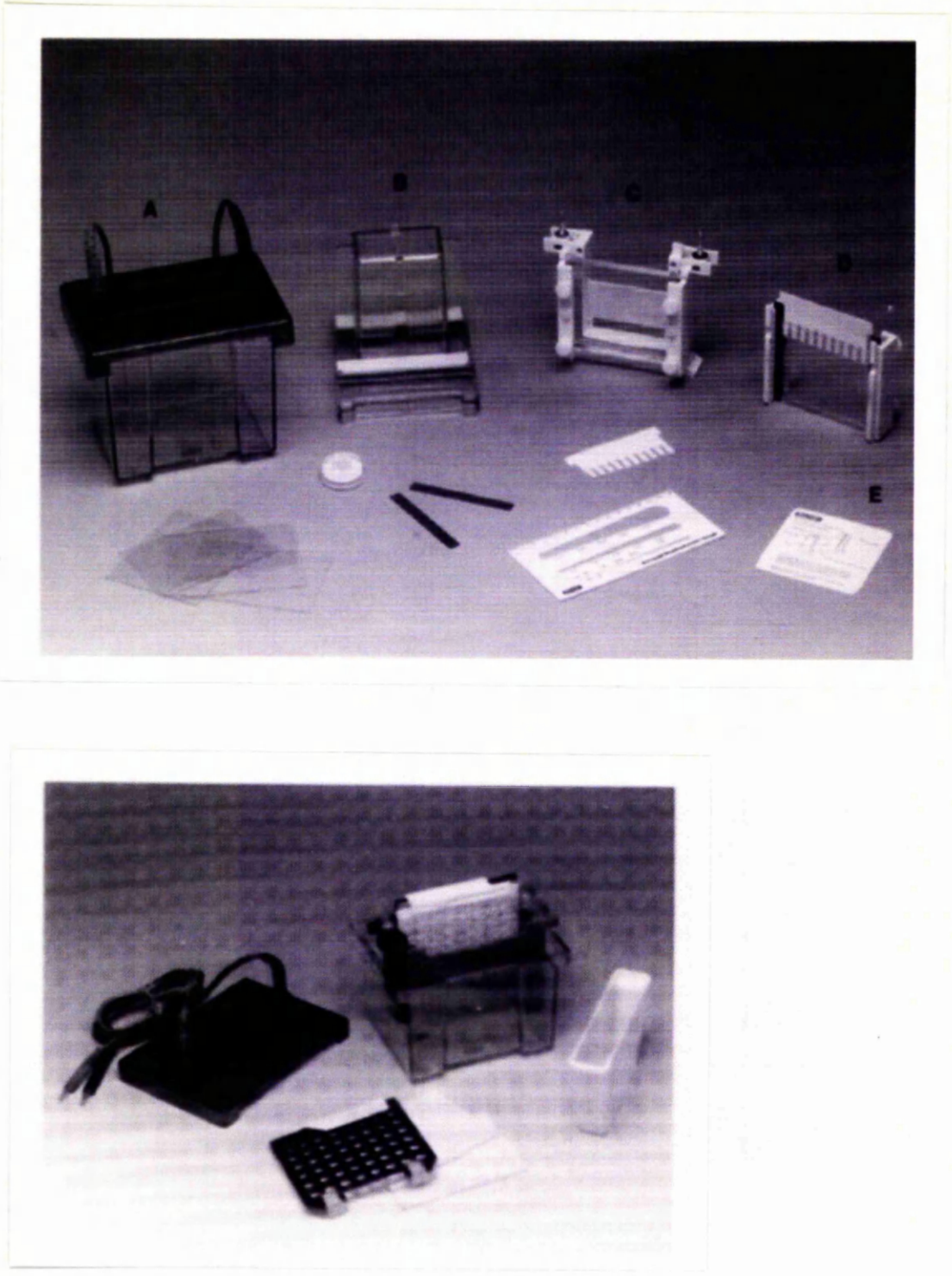


Figure 3.1

Photographs of Biorad MINI-PROTEAN II Dual slab gel apparatus (above) and Biorad Mini Trans-Blot electrophorestic transfer cell (below). Lower buffer chamber and lid (A), casting stand (B), inner cooling core (C), sandwich clamp assemblies (D), casting alignment card (E)

Preparation of gels

Assembling glass plates

Glass plates were washed in distilled water and polished with methanol prior to assembly. The outer and inner glass plates were separated using 0.5mm thick spacer bars and then fixed in the gel clamp assembly. These were then connected to the casting stand.

Casting the gels

Discontinuous polyacrylamide gels consisted of resolving or separating (lower) gels and stacking (upper) gels. Molecules were separated in the resolving gels using the Laemmli discontinuous buffer system. The running gel was prepared as below. The gel was allowed to polymerise for 1 hour and the iso-butanol removed. The top of the gel was then washed with distilled water and dried using a filter paper.

Running gel 0.375M Tris, pH 8.8

Reagent	12% gel	7.5% gel
Distilled water	3.35ml s	4.85mls
1.5M Tris HCL, pH8.8	2.5mls	2.5mls
10% SDS	100µl	100µl
Acrylamide/Bis (30%)	4mls	2.50mls
10% fresh NH^4 Persulphate	50µl	50µl
Tetramethylethylenediamine (TEMED)	5µl	5µl

The stacking gel was prepared and poured between glass plates on top of the running gel. A ten toothed comb (0.5cm spacing) was then placed into the running gel which was allowed to set for 1 hour.

Stacking Gel 4%, 0.125M Tris, pH 6.8

Distilled water	6.1mls
1.5M Tris HCL, pH8.8	2.50mls
10% SDS	100µl
Acrylamide (30%)	1.3mls
10% fresh NH^4 Persulphate	50µl
TEMED	5µl

The comb was then removed and wells washed with distilled water and dried in filter paper.

Assembling the electrophoresis cell

The clamp assemblies/gel sandwiches were released from the casting stand and then clamped onto the inner cooling core. This was lowered into the lower buffer chamber of the Mini-PROTEAN II cell. 300mls of electrode buffer (5x electrode buffer pH 8.3: 9gms Tris-base, 43.2gms glycine, 3gms SDS made up to 600mls with distilled water) was prepared. Approximately 115mls was then added to the upper chamber and the rest to the lower chamber.

Sample preparation

Samples were thawed slowly on ice. Appropriate volumes containing 100μg total protein were freeze dried, diluted with 10μl of reducing sample buffer (4mls distilled water, 1ml 0.5M Tris-HCL pH 6.8, 0.8ml glycerol, 1.6mls 10% SDS, 0.2ml 0.05% bromophenol blue, 50μl/ml mercaptoethanol) and boiled for two minutes. Gels were then loaded: lane 1 - molecular weight markers (Sigma, Dorset, UK), lane 2 - positive control, lanes 3 to 7 - breast tissue homogenates. Gels were electrophoresed for 1 hour at 20mA.

Protein transfer

Separated proteins were transferred to a nitrocellulose membrane using the Mini Trans-Blot electrophoretic transfer cell (Bio-Rad). Millipore nitrocellulose membrane

(Millipore, Bedford, UK) and 4 squares of 3MM paper were cut to the same size as the gel. The membrane was first soaked in methanol and then washed with distilled water. The membrane, paper and sponges soaked in transfer buffer (2.52gms sodium bicarbonate, 0.954gm sodium carbonate, 600mls industrial ethanol, 2.4L distilled water) for 30 minutes. The gel holder cassette was assembled incorporating gel and membrane. This was placed in the buffer tank and filled with transfer buffer. The assembly was placed in ice and run at 100kV, 400mA, 300W for 1 hour.

Immunostaining of membranes

Membranes were blocked using 5% non-fat dry milk (Marvel) diluted with 0.1% Tween 20 (polyoxyethylenesorbitan) for 1 hour. This was washed three times in Tris buffered saline (TBS) and the primary *c-met* specific antibody, 19S, was added (donated by Dr. G.F. Vande Woude, NCI, Frederick, USA). This antibody is monoclonal and raised against a 50kDa protein (p50met) from the carboxy-terminal part of the human *c-met* protein [93]. The antibody was used at a concentration of 1/1000 diluted in PBS/0.1% Tween 20 and incubated at room temperature overnight [256]. The membrane was washed three times in PBS/0.1% Tween 20 for 10 minutes. A secondary alkaline phosphatase coupled rabbit anti-mouse antibody (Dako, Glostrup, Denmark) was added at a concentration of 1/1000 diluted in PBS/0.1% Tween 20 and incubated at room temperature overnight. Membranes were washed twice in PBS/0.1% Tween 20 and then once in TBS pH 9.5 for 10 minutes. Bound antibody was visualised by a chromogenic reaction catalysed by the conjugated alkaline phosphatase using nitroblue tetrazolium

and 5-bromo-4-chloro-3-indoyl phosphate as chromogenic substrate. *C-met* expression was then visually assessed by an independent observer.

Controls

Controls included incubation of membrane without primary antibody and incubation with primary antibody which had been preincubated for 1 hour at room temperature with the competing peptide pmet 50 (1μg peptide per 1μl antibody, Dr.G.F. Vande Woude).

HGF/SF expression by enzyme linked-immunosorbent assay (ELISA)

HGF/SF levels in breast tissue homogenates were measured using a sandwich type ELISA developed between University Department of Pathology, University of Glasgow and Genentech Inc., San Francisco, USA. 96 microwell plates were coated with A3.1.2 HGF/SF specific mouse monoclonal antibody (Genentech Inc.) diluted in 0.5M sodium carbonate/0.5M sodium bicarbonate buffer at a concentration of 5ng/ml (100μl per well) and incubated for 16 hours at room temperature. The plate was washed six times with 0.05% Tween 20 in PBS and then blocked with 0.5% BSA/0.5% Tween 20 in PBS for two hours at room temperature. After washing six times with 0.05% Tween 20 in PBS each well was coated with 50μl of either test samples or standard concentrations of recombinant HGF/SF (Genentech Inc.) and incubated for 2 hours at room temperature. The plate was washed six times with 0.05% Tween 20 in PBS and each well coated

with sheep polyclonal anti-HGF/SF antibody (Genentech Inc.) at a concentration of 1/5000 in 0.5% BSA/PBS and incubated at room temperature for 1 hour. The plate was washed in 0.05% Tween 20 in PBS and each well coated with biotinylated donkey anti-sheep antibody (ICN Biomedicals, Thame, UK) at a concentration of 1/5000 in 0.5% BSA/PBS and incubated for 1 hour at room temperature. The plate was washed with 0.05% Tween 20 in PBS and then each well was coated with Avidin-Horseradish Peroxidase Conjugate at a concentration of 1/6000 and incubated for 1 hour at room temperature. The plate was washed six times with 0.05% Tween 20 in PBS and each well coated with O-phenylenediamine in 0.2M citric acid/potassium hydroxide at pH 5 with 25µl of concentrated hydrogen peroxide added immediately before use. The reaction was then stopped by the addition of 2M sulphuric acid. The plate was read using an automated microplate optical colometric densitometer at 492nm. Samples of tissue homogenate and standard dilutions of HGF/SF were analysed either in duplicate or triplicate. HGF/SF concentrations were then read from a curve derived from HGF/SF standards (Figure 3.2). Serial dilutions of homogenates were performed in subsequent assays to ensure results lay within the linear part of the standard curve. The lower detection limit of this assay was 0.25ng/ml (intra-ELISA variation: 1-5%, mean 2%). The HGF/SF concentrations were then converted to nanogrammes (ng) per 100mg of total protein.

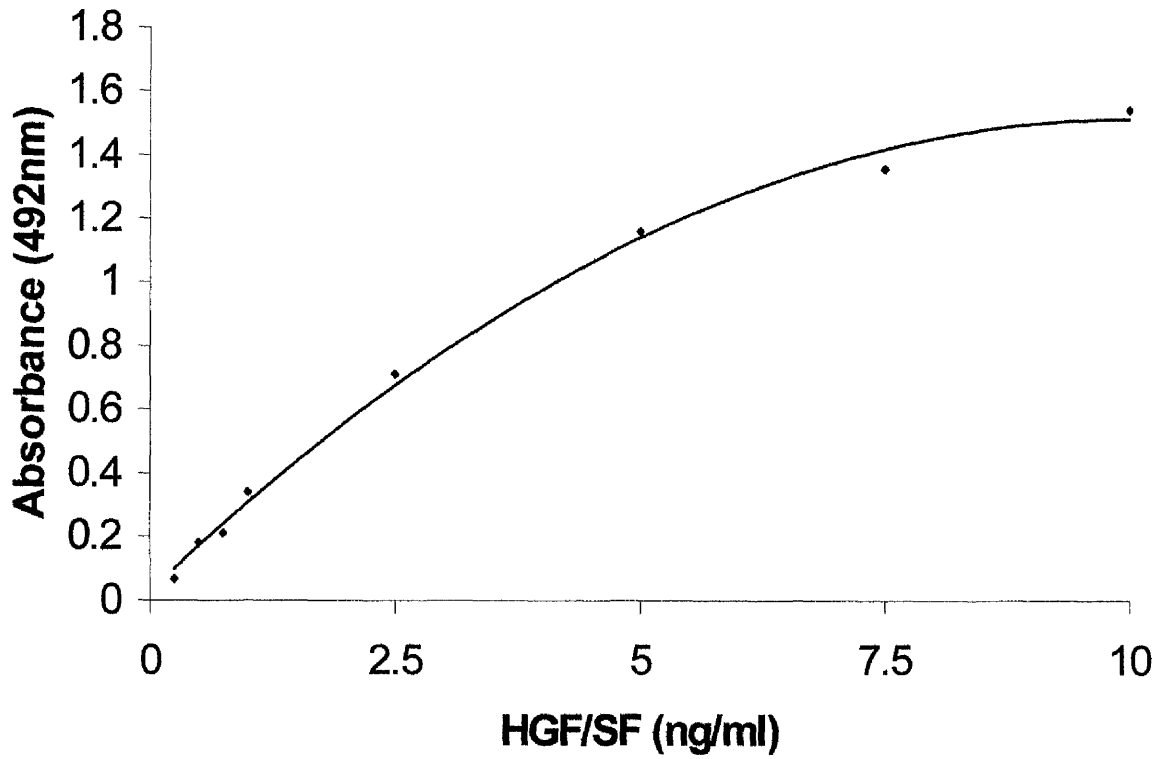


Figure 3.2

Typical HGF/SF standard curve. Absorbance at 492 nm (y axis), HGF/SF concentration in ng/ml (x axis).

A3.1.2 and sheep anti-HGF/SF specificity

Antibodies A3.1.2 and polyclonal sheep anti HGF/SF were checked for specificity and for cross reaction in breast cancer samples by Western blot electrophoresis on 12% polyacrylamide gels. Western blots of reduced samples of human recombinant HGF/SF and breast cancers were prepared. Membranes containing reduced HGF/SF were incubated overnight individually with A3.1.2 (1/2500) in PBS/0.1% Tween 20, sheep anti-HGF/SF (1/1000) in PBS/0.1% Tween 20 or preincubated antibody at room temperature. Preincubated antibodies were prepared by combining excess human recombinant HGF/SF (10μg) with either A3.1.2 (1/2500) or sheep anti-HGF/SF (1/1000) in PBS/0.1% Tween 20 overnight at room temperature. In addition non-specific protein binding by the A3.1.2 and sheep anti-HGF/SF antibodies were examined in six breast cancer samples by Western blot electrophoresis. Membranes treated with A3.1.2 were incubated with a secondary alkaline phosphatase coupled rabbit anti-mouse antibody (Dako, Glostrup, Denmark) at 1/10,000 in PBS/0.1% Tween 20 for 1 hour after washing. Membranes treated with sheep anti-HGF/SF were incubated with a secondary rabbit anti-sheep alkaline phosphatase coupled antibody (Dako, Glostrup, Denmark) at 1/5000 for 1 hour. Membranes were washed twice in PBS/0.1% Tween 20 and then once in TBS pH 9.5 for 10 minutes. Bound antibody was visualised by a chromogenic reaction catalysed by the conjugated alkaline phosphatase using nitroblue tetrazolium and 5-bromo-4-chloro-3-indoyl phosphate as chromogenic substrate.

Statistical analysis

The relationship between HGF/SF, prognostic factors and disease-relapse or overall survival could not be regarded as normally distributed and non-parametric statistical analysis was applied. They were tested by Mann-Whitney or Kruskal-Wallis tests for categorical prognostic factors or by Spearman's rank correlation for continuously variable prognostic factors. The relationship between *c-met* expression and clinico-pathological variables were tested using Chi-square or Mann-Whitney tests. The multivariate relationships between prognostic factors and disease-relapse were tested by analysis of variance and covariance.

Results (appendix 1)

C-met expression in non-malignant breast tissues and in breast cancer

Confirmation of antibody efficacy

Reduced *c-met* protein on Western blot electrophoresis is characterised by two protein bands of 170 and 145 kDa, with the 145 kDa band being the most prominent (170kDa band corresponding the precusror of p145 Met and the 145kDa band corresponding to the β-chain of the *c-met* receptor, p145 Met) [88]. Western blot analysis of the HT29 colon carcinoma cell line known to express *c-met* demonstrated the expected protein bands when using the 19S antibody (Figure 3.3). Incubation of the antibody with its competing peptide completely abolished binding to these proteins thus confirming specificity (Figure 3.3).

C-met expression in benign breast tissue

Ten samples of histologically proven benign breast tissue specimens were analysed for *c-met* expression. All samples demonstrated the two protein bands corresponding to *c-met* protein (Figure 3.4).

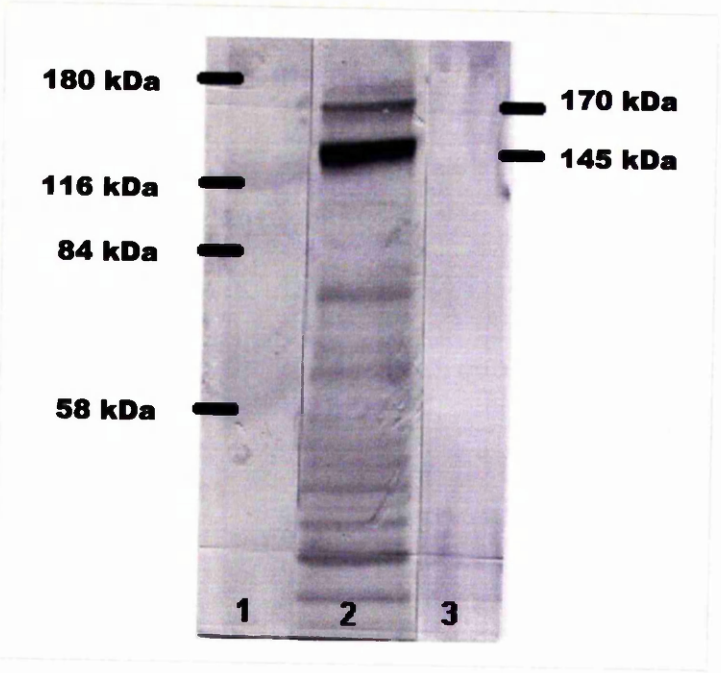


Figure 3.3

Western blot demonstrating *c-met* protein bands in HT 29 positive control. Lane 1: molecular weight markers. Lane 2: reduced HT 29 control. Two protein bands of 170 and 145 kDa are detected by 19S monoclonal antibody. Lane 3: Incubation of 19S antibody with its competing peptide completely abolishes binding to *c-met* proteins.

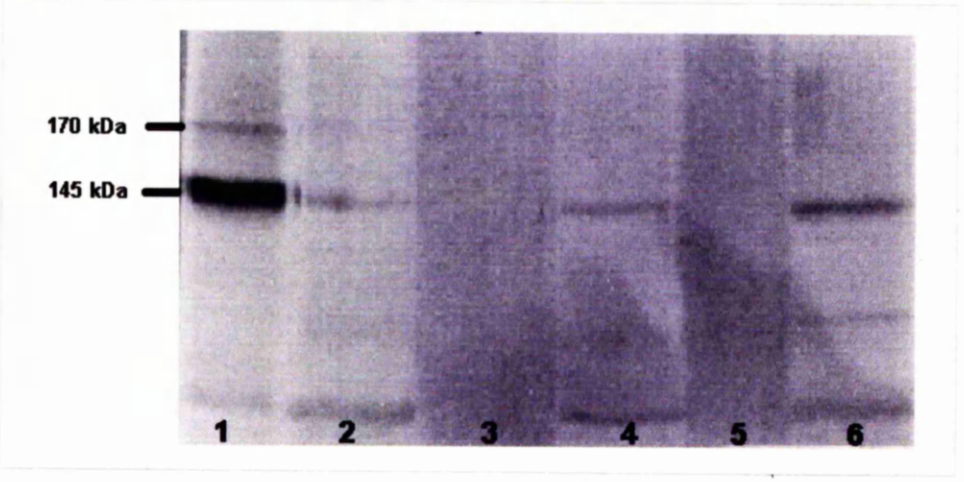


Figure 3.4

Representative Western blot of *c-met* protein in tumour-free breast tissue. Lane 1: HT 29 positive control. Lanes 2, 4 and 6: tumour-free breast tissue. Two *c-met* specific bands (p145 met and p170 met) were detected as labelled. Lanes 3 and 5 were blank.

C-met expression in breast cancer

C-met protein was detected in only 26 (36%) breast cancers, whereas in the remaining 47 (64%) breast cancers it was either absent or barely detectable (Figure 3.5). The relative levels of *c-met* expressed, comparing benign to malignant, could not be assessed by this method.

HGF/SF expression in non-malignant breast tissues and in breast cancer

Confirmation of antibody specificity and exclusion of cross-reactivity with tissue homogenates

Antibodies A3.1.2 and polyclonal sheep anti-HGF/SF were checked for specificity by Western blot electrophoresis of human recombinant HGF/SF. Both antibodies recognised the 69kDa heavy chain component of HGF/SF (Figure 3.6 and 3.7). Incubation of both antibodies with human recombinant HGF/SF eliminated binding to HGF/SF thus confirming antibody specificity (Figure 3.6 and 3.7). Absence of cross-reactivity between these antibodies and other tissue proteins was examined by Western blot analysis of several breast cancer homogenates. The A3.1.2 antibody demonstrated a high level of specificity and no cross reaction with other proteins confirming suitability for use as primary antibody for ELISA (Figure 3.8).

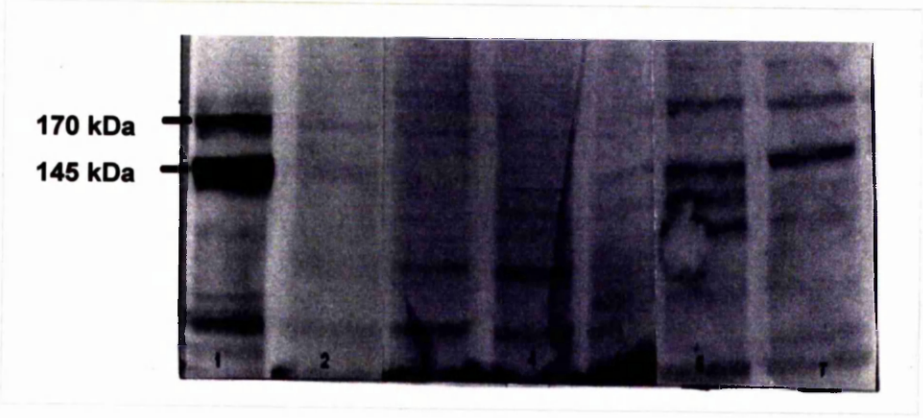


Figure 3.5

Representative Western blot of *c-met* protein in breast cancers. Lane 1: HT29 positive control. Lanes 2-7: breast cancers. Lanes 6 and 7: breast cancers with detectable *c-met* protein. Lanes 2, 3, 4 and 5: breast cancers with absent or barely detectable *c-met* protein. Two *c-met* specific bands (p145 met and p 170 met) were detected.

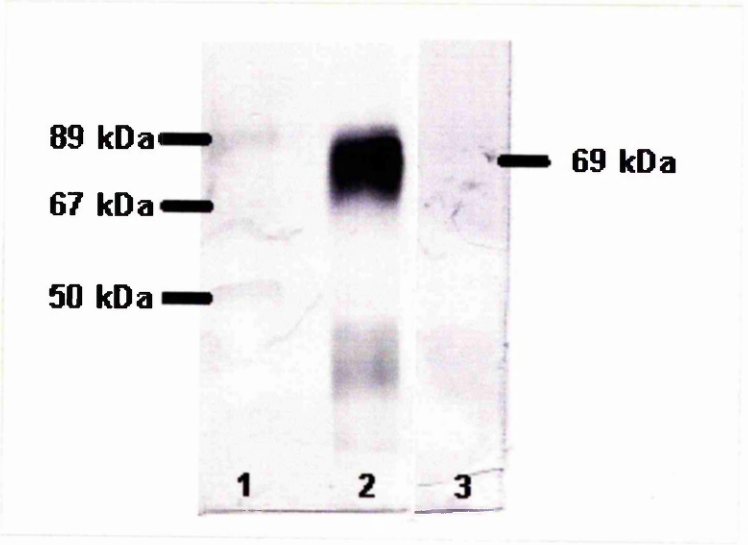


Figure 3.6

Western blot demonstrating 69 kDa band of reduced human recombinant HGF/SF detected by A3.1.2 monoclonal antibody. Lane 1: molecular weight markers. Lane 2: reduced HGF/SF. 69 kDa band corresponding to α-chain seen. Lane 3: pre-incubation of A3.1.2 with HGF/SF abolishes binding to HGF/SF α-chain thus confirming antibody specificity.

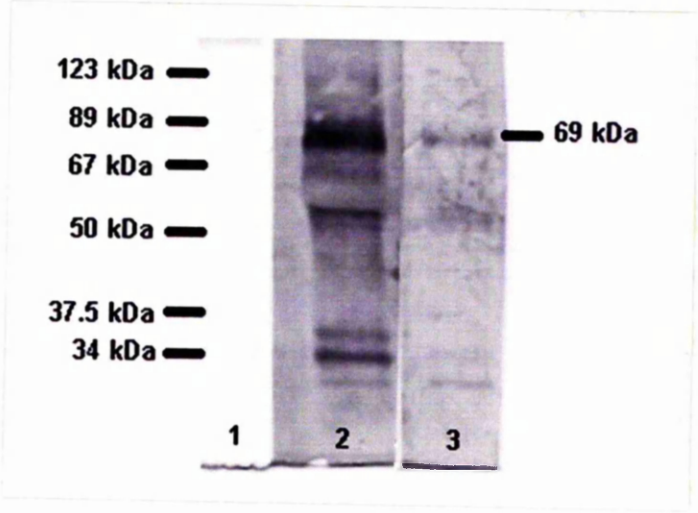


Figure 3.7

Western blot demonstrating 69 kDa band of HGF/SF derived from MRC-5 fibroblasts detected by sheep polyclonal HGF/SF antibody(Genentech). Lane 1: molecular weight markers. Lane 2: reduced HGF/SF. 69 kDa band corresponding to α-chain of HGF/SF. Lane 3: pre-incubation of sheep polyclonal HGF/SF antibody with HGF/SF reduces binding to HGF/SF α-chain. Other protein bands are also seen in lanes 2 & 3. This antibody was used to detect HGF/SF bound to A3.1.2 in ELISA and would not be suitable for use as primary antibody.

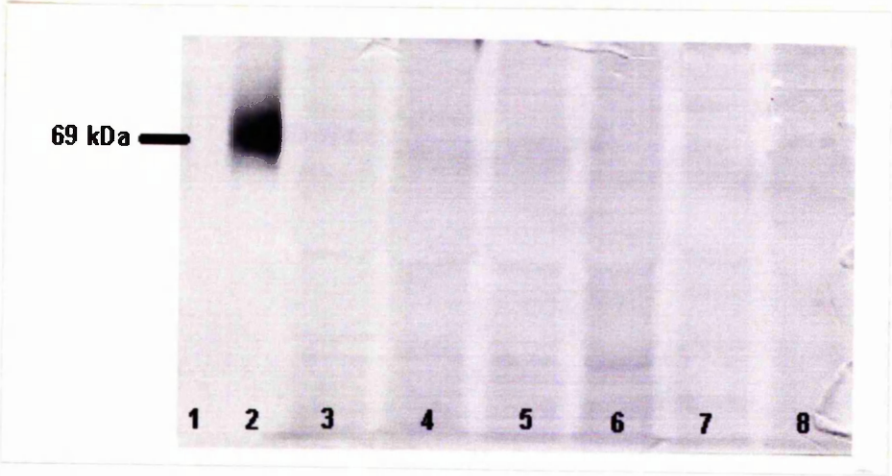


Figure 3.8

Western blot of breast cancer homogenates with A3.1.2 monoclonal antibody (Genentech). Lane 1: molecular weight markers. Lane 2: reduced HGF/SF demonstrating 69 kDa α-chain. Lanes 3-8: reduced breast cancer homogenates. No protein bands detected. Although breast cancer homogenates contain HGF/SF as detected by ELISA, levels may be too low to detect by Western blot. However, this experiment provides some evidence of A3.1.2 specificity and absence of cross-reactivity with other protein species.

HGF/SF levels in breast cancer and tumour-free breast tissue

HGF/SF was detected in all samples of tumour-free breast tissue and in 71 breast cancers. The remaining two breast cancer samples were insufficient for duplicate or triplicate ELISA analysis. The concentration of HGF/SF was significantly higher in breast cancers (range 58-1604ng per 100mg total protein; mean: 396ng per 100mg total protein) than tumour-free breast tissue (range 66-200ng per 100mg total protein; mean: 130ng per 100mg total protein), $p<0.001$, Figure 3.9.

Prognostic significance of *c-met* and HGF/SF in breast cancer

Patient data

Clinico-pathological prognostic variables from 73 patients with breast cancer are summarised (Table 3.1). The median follow-up was 61 months (range 7-89 months). Local recurrence was observed in three patients, local and systemic recurrence in nine patients and systemic recurrence in 16 patients. All patients with systemic recurrence died from metastatic disease. Positive nodal status correlated with disease-relapse ($p=0.013$) and reduced overall survival ($p=0.024$), (Table 3.2). Tumour size correlated with disease-relapse ($p=0.027$), (Table 3.2). The remaining prognostic clinico-pathological variables did not correlate with either disease-relapse or overall survival (Table 3.2).

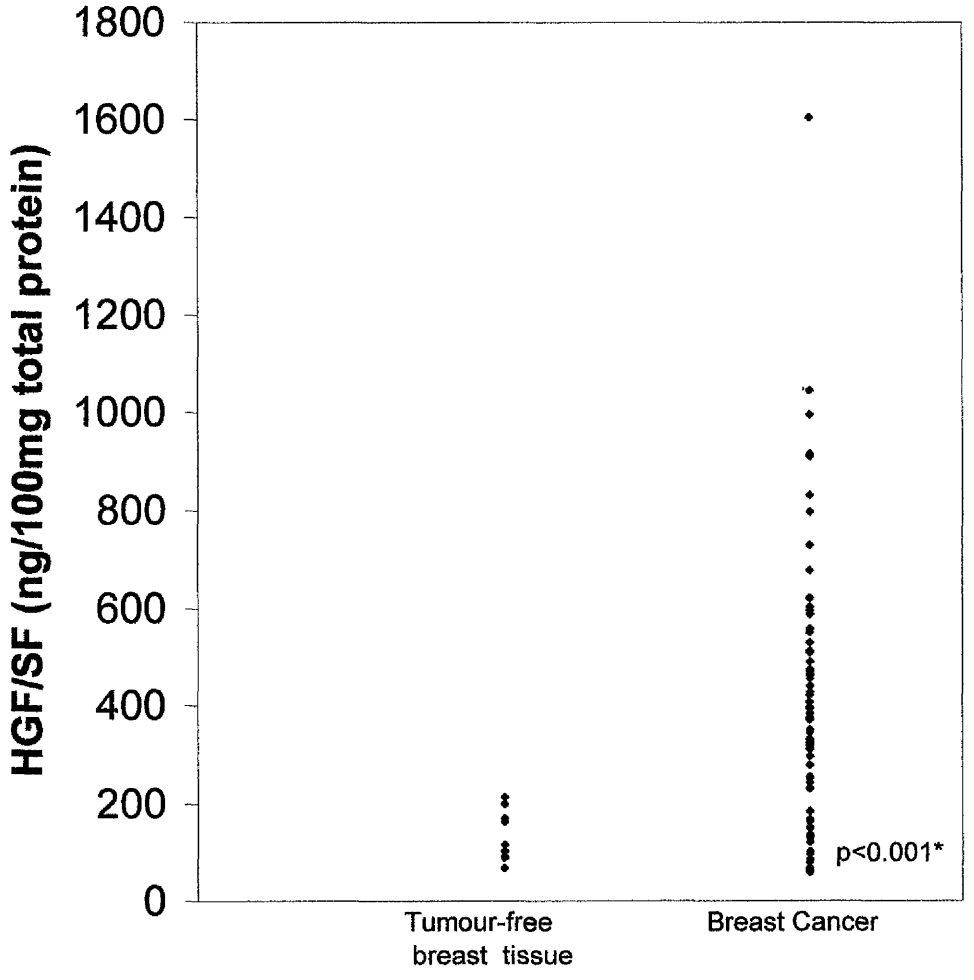


Figure 3.9

Scatterplot comparing HGF/SF levels in tumour-free breast tissue and breast cancers.

* statistically significant (Spearman's rank correlation)

Table 3.1

Clinico-pathological prognostic variables of 73 patients with breast cancer.

Prognostic variables	**n**
Age (years)	
<55	28
≥55	45
Tumour size (mm)	
<25	26
≥25	47
Lymph node status*	
Positive	36
Negative	33
Tumour Grade **	
1	8
2	31
3	27
Oestrogen receptor status***	
Positive	29
Negative	32
Lymphatic Invasion	
Present	21
Absent	52

* Lymph node status unknown in 4 patients

** Tumours not graded (4 lobular, 2 mucoid, 1 medullary)

*** Oestrogen receptor status unknown in 12 patients

Table 3.2

Categorical prognostic factors and tests of their relationship with disease-free and overall survival. The prognostic value of dichotomous factors (*c-met* expression, nodal status, tumour size, oestrogen receptor, lymphatic invasion) were tested by the Mann-Whitney test and the prognostic value of histological grade was tested by the Kruskal-Wallis test. Continuously variable prognostic factors: HGF/SF, age and tumour size were tested by Spearman's rank correlation coefficient corrected for ties.

Prognostic factor	**Values**	**n**	**Median Relapse-free survival (months)**	**p value**	**Median overall survival (months)**	**p value**
c-met	Negative Positive	47 26	>60 42.5	0.012	>60 60	0.080
Nodal status	Negative Positive	33 36	>60 46	0.013	>60 60	0.024*
Histological grade	1 2 3	8 31 27	>60 >60 >60	0.369	>60 >60 >60	0.205
Oestrogen receptor	Negative Positive	31 30	>60 >60	0.262	>60 >60	0.238
Lymphatic invasion	Negative Positive	52 21	>60 >60	0.427	>60 >60	0.472

Prognostic factor	**n**	**Correlation to disease-free survival**	**p value**	**Correlation to overall survival**	**p value**
HGF/SF	71	-0.4916	<0.001	-0.4258	<0.001*
Age (years)	73	-0.1185	0.318	-0.0645	0.588
Tumour size (mm)	73	-0.2597	0.027	-0.1862	0.115

* statistically significant

HGF/SF levels not available in 2 patients
Lymph node status unknown in 4 patients
Tumours not graded (4 lobular, 2 mucoid, 1 medullary)
Oestrogen receptor status unknown in 12 patients

HGF/SF and *c-met* expression in breast cancer

Univariate analysis

HGF/SF concentrations demonstrated significant correlation with both disease-relapse and overall survival in patients with breast cancer. Levels in patients with disease-relapse (median 502ng/100mg total protein) were significantly higher than those who remained disease-free (median 297ng/100mg total protein), $p<0.001$, (Figure 3.10). Similarly, HGF/SF levels were significantly increased in patients who died from disease progression (median 513ng/100mg total protein) when compared to survivors (median 313ng/100mg total protein), $p<0.001$, (Figure 3.11). There was a non-significant trend between increasing HGF/SF levels and poorer histological grade (Figure 3.12). However, HGF/SF levels did not show any correlation with nodal status, tumour size, oestrogen receptor status and lymphatic invasion (Table 3.3). HGF/SF levels appeared to be higher in tumours expressing *c-met* (*c-met* negative: median 330ng/100mg total protein; *c-met* positive: median 407ng/100mg total protein).

The statistical relationships between *c-met* expression, survival and clinico-pathological variables are summarised (Tables 3.2 and 3.4). Breast cancers with detectable *c-met* protein demonstrated a significant association with disease-relapse, $p=0.012$, (Figure 3.13). There were non-significant associations between *c-met* with reduced survival, $p=0.080$, (Figure 3.14) and *c-met* with lymphatic invasion ($p=0.057$). There were no significant relationships between *c-met*, nodal status, tumour grade, tumour size and

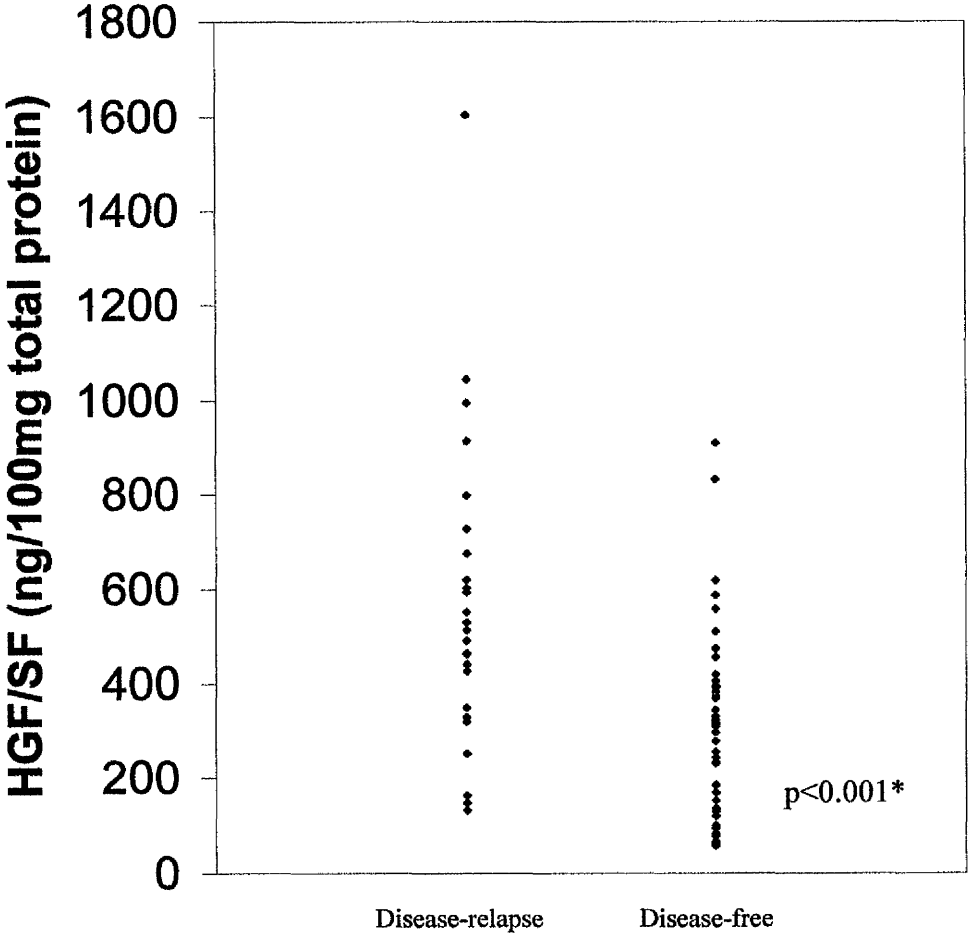


Figure 3.10

Comparison of tumour HGF/SF levels in patients with disease-relapse to those remaining disease-free.

* statistically significant (Mann-Whitney)

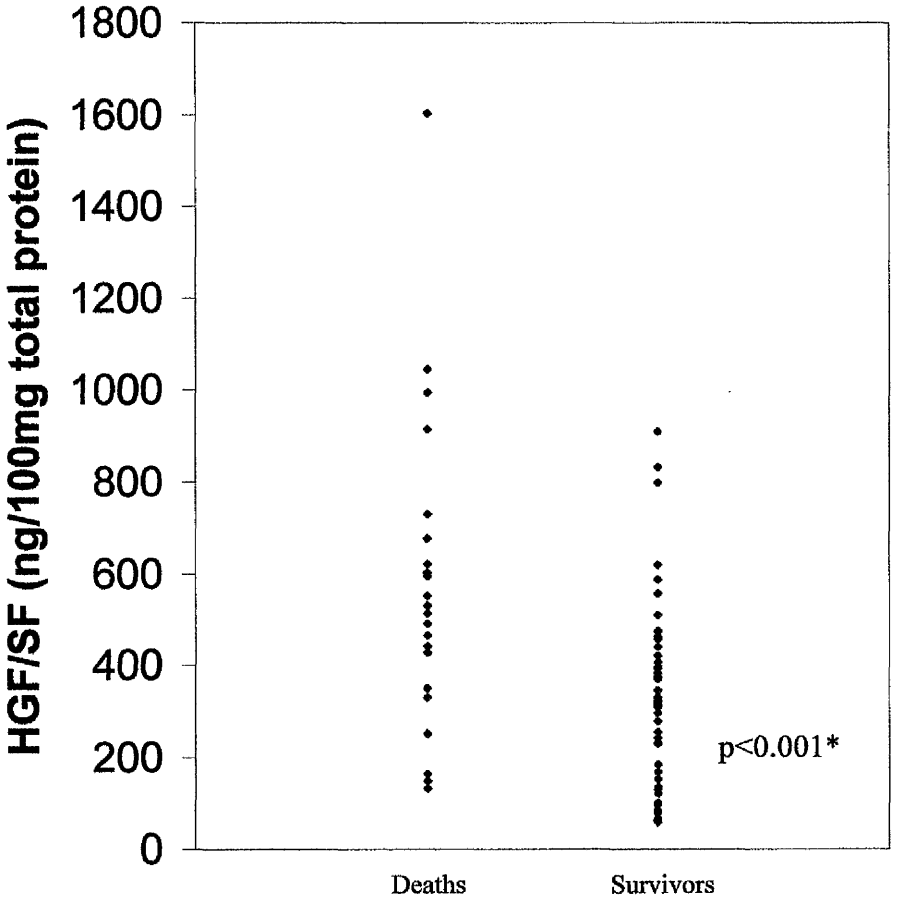


Figure 3.11

Comparison of HGF/SF levels with survival in patients with breast cancer.

* statistically significant (Spearman's rank correlation)

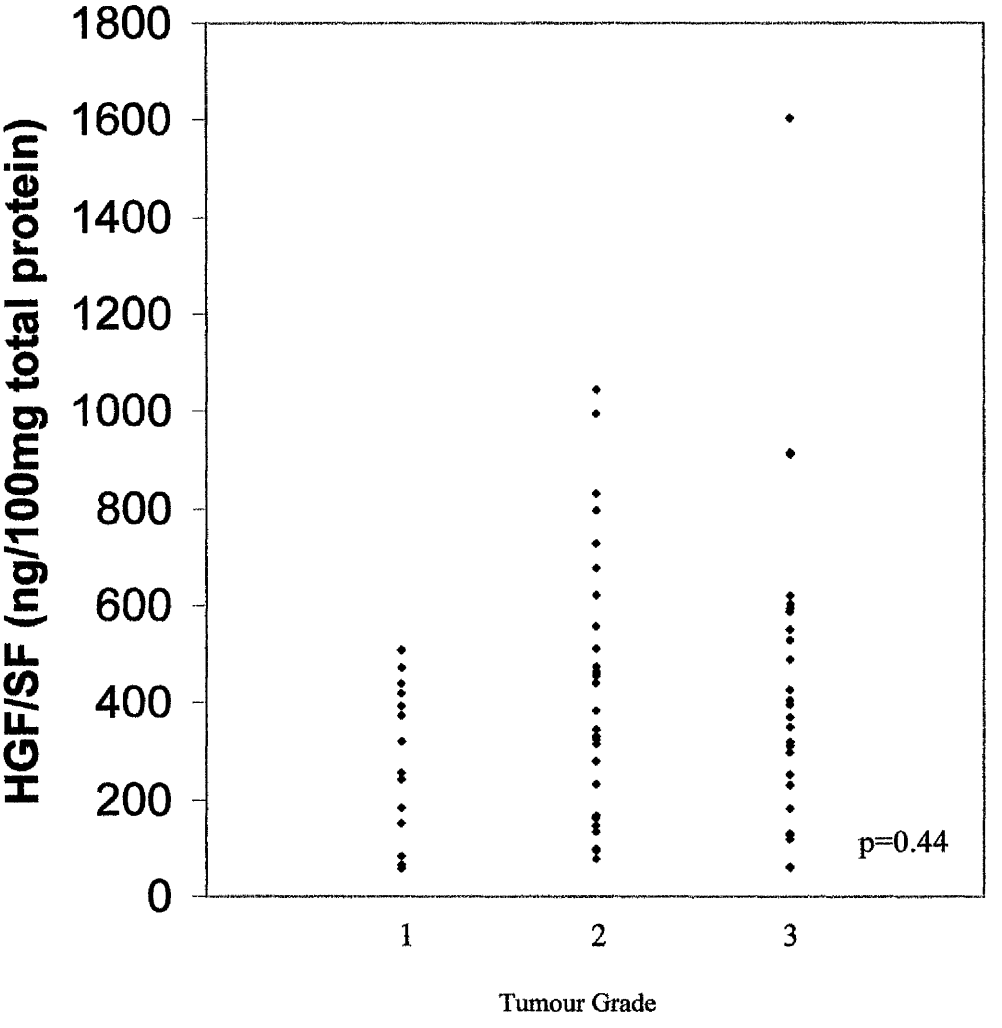


Figure 3.12

Comparison of tumour HGF/SF levels with histological grade in patients with breast cancer (p=0.44, Kruskal-Wallis).

Table 3.3

Categorical prognostic factors and tests of their relationship with HGF/SF. The relationship of nodal status, oestrogen receptor and lymphatic invasion to HGF/SF was tested by the Mann-Whitney test. The relationship of histological grade to HGF/SF was tested by the Kruskal-Wallis test. Continuously variable prognostic factors, age and tumour size, were tested by Spearman's rank correlation coefficient corrected for ties.

Prognostic factor	**Values**	**n**	**Median HGF/SF level**	**p value**
Nodal status	Negative	33	378.0	0.99
	Positive	36	350.0	
Histological grade	1	8	252.5	0.44
	2	31	345.0	
	3	27	370.0	
Oestrogen receptor	Negative	31	374.0	0.87
	Positive	30	388.0	
Lymphatic invasion	Negative	52	338.0	0.53
	Positive	21	370.0	

Prognostic factor	**n**	**Correlation with HGF/SF**	**p value**
Tumour size	71	0.024	0.84
Age	71	0.071	0.54

HGF/SF levels not available in 2 patients
Lymph node status unknown in 4 patients
Tumours not graded (4 lobular, 2 mucoid, 1 medullary)
Oestrogen receptor status unknown in 12 patients

Table 3.4

Categorical prognostic factors and tests of their relationship with *c-met*. The relationship of nodal status, tumour grade, oestrogen receptor and lymphatic invasion to *c-met* status was tested by the Chi-squared test with Yates modification. The relationship between *c-met* to age and tumour size was tested using the Mann-Whitney test.

Variable	**n**	**Number *c-met* negative**	**Number *c-met* positive**	χ^2	**p value**
Nodal status	Negative Positive	24 20	9 16	2.197 1 df	0.14
Tumour grade	1 2 3	5 19 12	3 12 8	0.017 2df	0.76
Oestrogen receptor	Negative Positive	18 20	11 10	0.136 1df	0.86
Lymphatic invasion	Negative Positive	37 10	15 11	3.613 1df	0.057

Prognostic factor	**Values**	**n**	**Median age**	**p value**
Age	*c-met* negative *c-met* positive	47 26	62 56	0.52

Prognostic factor	**Values**	**n**	**Median tumour size**	**p value**
Tumour size	*c-met* negative *c-met* positive	47 26	28 30	0.89

Lymph node status unknown in 4 patients
Tumours not graded (4 lobular, 2 mucoid, 1 medullary)
Oestrogen receptor status unknown in 12 patients

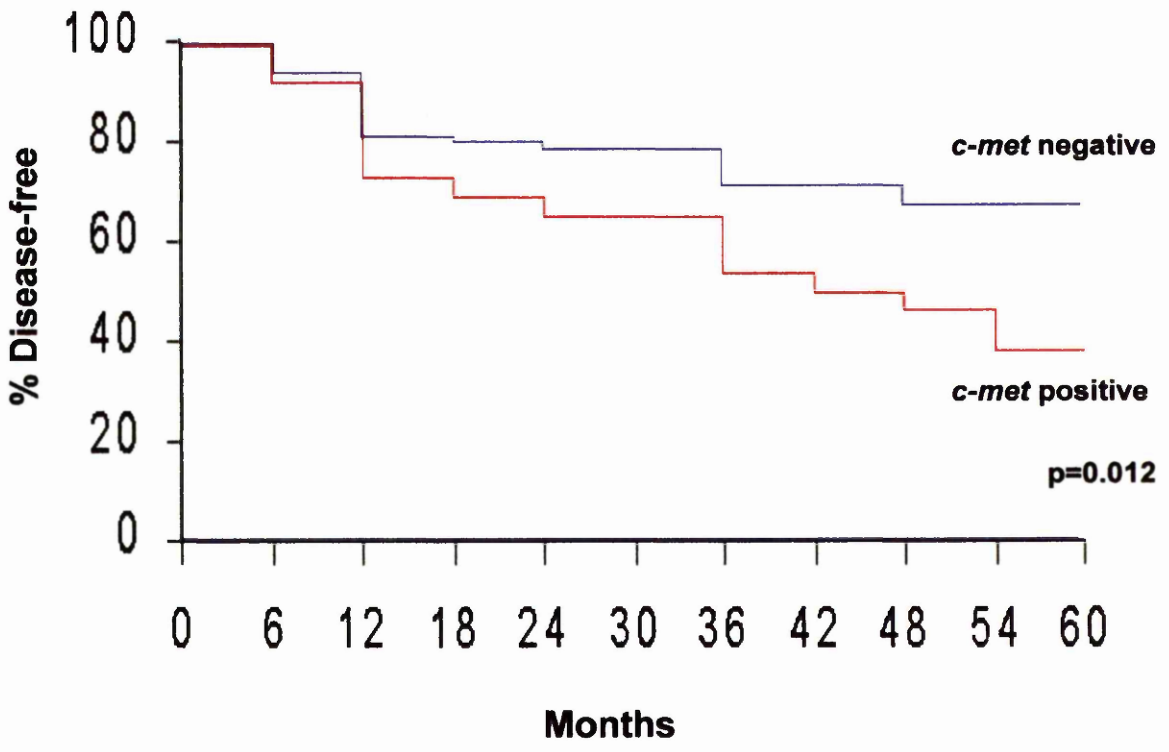


Figure 3.13

Graph of relationship between *c-met* expression and disease-relapse in patients with breast cancer (p=0.012, Mann-Whitney).

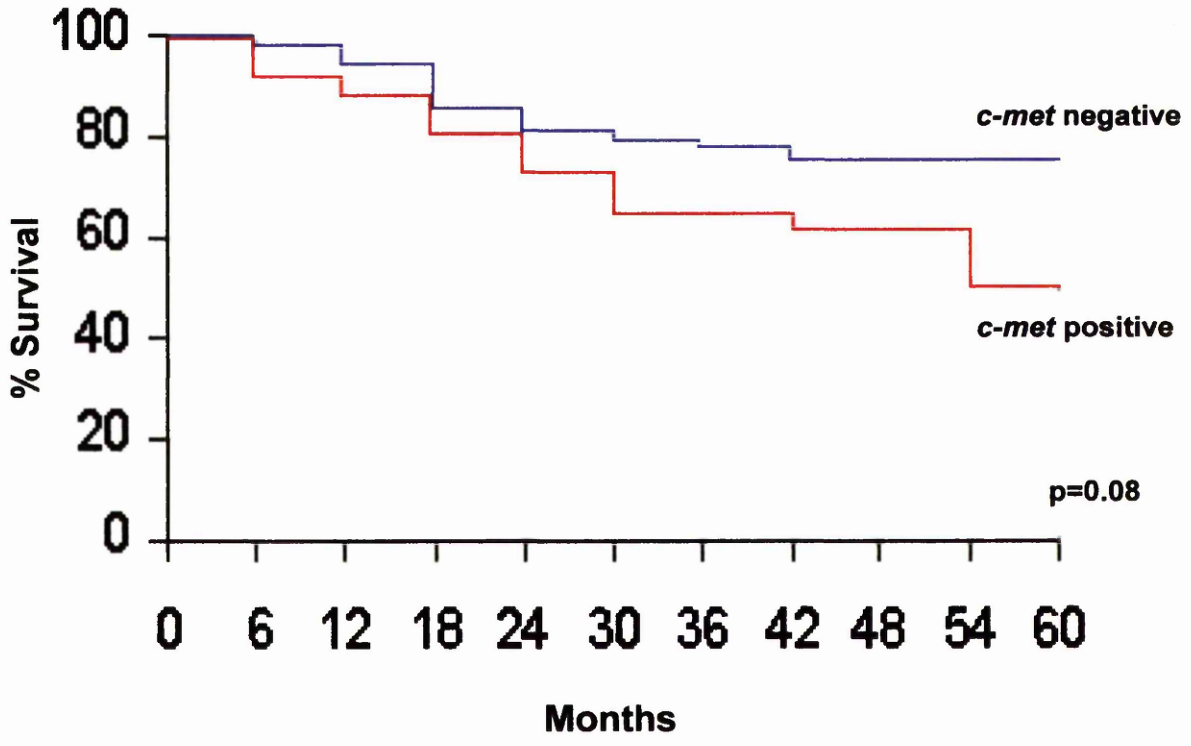


Figure 3.14

Graph of relationship between *c-met* expression and overall survival in breast cancer (p=0.08, Mann-Whitney).

oestrogen receptor status.

Relationship between HGF/SF, *c-met*, prognostic variables and disease-relapse

Non-linear discriminant analysis combined with analysis of variance and covariance suggested a statistical interaction between HGF/SF and *c-met* in relation to disease-relapse (appendix 3). Non-linear discriminant analysis was carried to predict disease-free survival. Initially the variables; age, size, grade, nodal status, oestrogen receptor status, lymphatic invasion, *c-met*, HGF/SF and the combined term HGF/SF**c-met* were considered for inclusion in the model. Using Wilks' lambda method, these prognostic indicators were tested for any statistically significant contribution to the accuracy of prognosis. Only three prognostic factors satisfied entry into the model; HGF/SF**c-met* ($p=0.0001$), HGF/SF ($p<0.001$) and nodal status ($p<0.001$). Importantly, the effect of *c-met* independent of HGF/SF became statistically non-significant in relation to disease-relapse. This suggests that the observed effect of *c-met* on disease-relapse is wholly dependant on the presence of HGF/SF.

In the analysis of variance and covariance, the prognostic variables: age, tumour size and grade, nodal status, oestrogen receptors, lymphatic invasion, HGF/SF and *c-met* were tested for the statistical significance of their relationship to disease-relapse. Any insignificant terms were removed from the model sequentially. On introduction of the HGF/SF**c-met* interaction term, the effect of *c-met* became insignificant. In this model, the most significant predictors of disease-relapse were HGF/SF ($p<0.001$) and the

interaction of HGF/SF and *c-met* (p=0.014). Other predictors of disease-relapse were tumour size (p=0.037) and nodal status (p=0.054).

These multivariate analyses demonstrate a significant statistical interaction between HGF/SF and *c-met* in relation to disease-relapse. When this interaction (HGF/SF**c-met*) is added to the above models, the direct effect of *c-met* alone became insignificant suggesting the effects of *c-met* are entirely due to HGF/SF. Therefore this observation provides strong support of a ligand-receptor interaction promoting disease-progression in human breast cancer.

Discussion

Aberrant HGF/SF-*c-met* expression has been shown to induce tumour invasion and metastasis both *in vitro* and *in vivo*. This study provides support for this hypothesis in human breast cancer. Multivariate analysis demonstrated a statistically significant interaction between HGF/SF and *c-met* in relation to disease-relapse. In addition, HGF/SF levels were increased in breast cancer and tumour levels of HGF/SF correlated with disease-relapse and reduced survival. Furthermore, breast cancers with detectable *c-met* demonstrated a significant correlation with disease-relapse.

HGF/SF levels are increased in both breast cancer homogenates and in sera of patients with breast cancer [294,331,332,335]. In this study we observed significantly increased HGF/SF levels in breast tumour homogenates when compared to tumour-free breast tissue. Tumour levels of HGF/SF correlated with significant reduction in both disease-free and overall survival. These results have been confirmed by other studies. Yamashita *et al* observed that HGF/SF levels were significantly increased in breast cancer samples when compared to normal tissue [331]. Furthermore, a powerful correlation between increased HGF/SF levels and reduction in disease-free and overall survival was observed in a study of 248 patients with breast cancer [332]. In addition, HGF/SF levels only correlated to tumour size and were otherwise independent of other prognostic factors. Similarly, Yao *et al* observed HGF/SF levels were significantly increased in breast cancers when compared to normal breast tissue [335]. In their study node positive breast cancers expressed significantly higher levels of HGF/SF when

compared to node negative disease and HGF/SF levels were also related to tumour size. Serum levels of HGF/SF are also increased in breast cancer patients. Taniguchi *et al* detected significantly increased HGF/SF levels in 36.6% of breast cancer patients [294]. This observation was confirmed by Toi *et al* who also observed that increased levels of HGF/SF correlated with axillary nodal status and evidence of venous invasion [301]. Circulating levels of HGF/SF decreased when primary tumour was removed suggesting that the observed elevated HGF/SF level was tumour-related [301].

Detectable *c-met* in breast cancer is associated with poorer prognosis. This study has demonstrated a significant reduction in disease-free survival in breast cancers with detectable *c-met* protein. This observation has been confirmed by Ghoussoub *et al* who reported that tumour expression of *c-met* correlated with a significant reduction in 5 year survival in patients with breast cancer [86]. This association was seen in both node negative and node positive groups. Multivariate analysis demonstrated that *c-met* expression was an independent predictor of survival.

Expression of *c-met* protein appears to be reduced in the majority of breast cancers when compared to tumour-free breast tissue. In this study *c-met* was detected in all samples of tumour-free breast tissue. However, *c-met* was barely detectable or absent in the majority of breast cancers. This observation is similar to Tsarfaty *et al* who observed higher levels of *c-met* expression in adjacent tumour-free ductal breast epithelium when compared to tumour [303]. Furthermore, Ghoussoub *et al* detected moderate-to-intense *c-met* expression in normal ductal breast epithelium where only

22% of 91 breast cancers expressed *c-met* [86]. These observations suggest that *c-met* expression is down-regulated in a large proportion of breast cancers. A reciprocal relationship between HGF/SF and *c-met* expression may exist in normal breast tissue and breast cancers. Normal breast tissue is characterised by low levels of HGF/SF expression and high levels of *c-met*. However, the majority of breast cancers exhibit high levels of HGF/SF and low levels of *c-met* expression. HGF/SF has been observed to down-regulate *c-met* expression in primary fibroblast cell cultures via an extracellular-autocrine pathway [240]. It is possible that increased levels of HGF/SF in breast cancers *in vivo* down-regulate *c-met* expression in well differentiated but not in poorly differentiated cancers. This hypothesis is supported by the observation that *in vitro c-met* expression is low in well differentiated and high in poorly differentiated breast cancer cell lines [26]. However this series failed to detect any relationship between *c-met* expression and tumour grade ($p=0.76$, Table 3.3).

Although the biological actions of HGF/SF are transduced through *c-met*, this is the first study to report a relationship between this ligand-receptor pair in relation to disease progression. Studies both *in vitro* and *in vivo* have shown that HGF/SF activation of *c-met* is both tumorigenic and a promoter of tumour invasion and metastasis [115]. The results of this study provide strong support for the hypothesis that aberrant HGF/SF-*c-met* expression plays a role in disease progression in breast cancer. Multivariate analysis demonstrated a statistically significant interaction between HGF/SF and *c-met* expression in relation to disease-relapse. This observation suggests that breast cancer progression may in part be due to ligand-driven tumour invasion and metastasis through

HGF/SF-*c-met*. The implications of these findings are not only of prognostic utility, antagonism of HGF/SF-*c-met* may have therapeutic benefits.

In vitro studies suggest a paracrine mode of interaction between HGF/SF and *c-met*. Fibroblasts derived from breast tissue appear to produce HGF/SF whereas breast epithelial cells in culture do not express HGF/SF [13,268,280]. However, studies *in vivo* observe HGF/SF expression in both epithelial and stromal components of breast tissue. Wang *et al* detected HGF/SF mRNA by in-situ hybridisation mainly in the epithelial component of normal and malignant breast tissues [317]. Tuck *et al* observed HGF/SF in both epithelial and stromal components of breast cancers whereas *c-met* expression was restricted to epithelial cells, using both in-situ hybridisation and immuno-histochemical techniques [307]. In addition, tumour cells that were strongly positive for HGF/SF were also strongly positive for *c-met* suggesting a possible autocrine loop effect. Finally, Ghoussoub *et al* detected *c-met* staining in epithelial component of both normal breast and breast cancers [86]. These studies show that HGF/SF-*c-met* may function in either autocrine or paracrine modes. Studies of HGF/SF-*c-met* expression in embryogenesis suggest the physiological mode of interaction is paracrine [275].

Autocrine HGF/SF-*c-met* expression is both highly tumorigenic and a powerful inducer of tumour invasion and metastasis. The creation of HGF/SF-*c-met* autocrine loops in transgenic mice has been shown to induce a variety of tumours with mammary glands being the most prevalent site [290]. Meiners *et al* transfected HGF/SF cDNA into the human MDA MB 435 mammary carcinoma cell line and then injected these into the

mammary fat pads of nude mice [176]. Non-transfected cells failed to metastasise whereas transfected breast cancer cells demonstrated strong growth and spontaneous metastasis to lung. Autocrine activation of HGF/SF-*c-met* may therefore represent an important pathological step in tumour metastasis.

There is mounting evidence that HGF/SF-*c-met* plays a central role in breast duct development. The possibility of HGF/SF being associated with breast ductal morphogenesis was suggested by Tsarfaty *et al* who observed breast cancer cells in culture forming lumen-like structures when exposed to the factor [303]. Berdichevsky *et al* demonstrated fibroblast induced branching morphogenesis of human mammary cells in collagen gels [13]. The factor responsible for this effect was identified as HGF/SF. In their report, factors such as acidic FGF, basic FGF and PDGF had no effect on ductal morphogenesis, whereas TGF-β inhibited HGF/SF induced branching. Similar findings were reported by Soriano *et al*, who identified HGF/SF as the fibroblast derived factor inducing ductal branching and lumen formation in murine mammary cells cultured in collagen gels [276]. HGF/SF was observed to increase cord length 11-22 times more potently than TGF-α and EGF. IGF-II, basic FGF and PDGF had no such effect and again TGF-β inhibited HGF/SF induced ductal morphogenesis. Systemic hormones such as oestrogen, progesterone, insulin and prolactin were not observed to induce branching morphogenesis. Although these factors are essential to breast development, final expression of their signal may require interaction of stromal cells through production of local factors. Yang *et al* demonstrated that HGF/SF promoted branching morphogenesis in post-natal mouse mammary organ cultures [334]. Finally, Niranjan *et al*

demonstrated HGF/SF induced branching morphogenesis in human mammary organoids embedded in collagen gels [208]. HGF/SF levels in developing breast appear to be strongly regulated. Yang *et al* observed high levels of HGF/SF expression during ductal morphogenesis in virgin mice, expression decreasing during pregnancy and lactation and increasing again during involution [334]. These observations were confirmed by Niranjan *et al* who detected HGF/SF in addition to *c-met* expression during mouse mammary gland development [208].

In conclusion, HGF/SF and *c-met* are important factors involved in both breast duct development and in tumour progression in breast cancer. This study provides evidence of a statistically significant interaction between HGF/SF and *c-met* in relation to disease-relapse. It is not known whether ligand-receptor interactions switch from paracrine to autocrine in tumour cells that undergo metastasis. However, autocrine HGF/SF-*c-met* loops *in vivo* strongly promote metastases in experimental systems. Agents that interfere with HGF/SF-*c-met* interactions may provide important new therapeutic agents in breast cancer treatment.

Chapter 4: Hepatocyte growth factor/scatter factor, angiogenesis and tumour cell proliferation in primary breast cancer

Introduction

Proliferation and angiogenesis are important prognostic variables in breast cancer. However, little is known regarding the intrinsic and extrinsic factors that control these variables. In breast cancer growth factors such as basic FGF, PDGF, TGF-α, VEGF, growth factor receptors to EGF and PDGF and the growth inhibiting factor TGF-β may be implicated [49]. HGF/SF expression is increased in human breast cancer. *In vitro* studies have shown that HGF/SF stimulates cell growth, dissociation, motility and invasiveness in a wide variety of epithelial cell types including breast [244,247,250]. Treatment of EMT6 mouse mammary tumour cells with HGF/SF stimulates invasion through matrigel, expression of soluble and cell surface urokinase and lung colonisation [247]. HGF/SF is also a potent inducer of angiogenesis with a nanogram potency similar to basic fibroblast growth factor [242]. Bussolino *et al* demonstrated human endothelial cell proliferation and motility *in vitro* and neovascularisation at sub-nanomolar concentrations in the rat cornea [25]. Grant *et al* similarly demonstrated HGF/SF induced vascular endothelial cell migration, proliferation and organisation into capillary-like tubes *in vitro*. Furthermore, HGF/SF induced angiogenesis *in vivo*, at pico-molar concentrations in the absence of inflammation in rat corneas [94]. HGF/SF and *c-met* mRNA have been isolated from human endothelial and vascular smooth muscle cells suggesting autocrine-paracrine interactions [200]. Tumour angiogenesis is a prerequisite

for tumour growth beyond 2-3mm^3 and for metastasis [72,73].

Tumour concentrations of HGF/SF have been shown to correlate with reduced relapse-free and overall survival in patients with primary breast cancer [332,335]. Clinical studies have shown that a high degree of neovascularisation is an indicator of poor prognosis in a wide range of human cancers including breast. Several studies have demonstrated correlation between indices of angiogenesis and survival in patients with breast and other cancers [324]. Tumour proliferation indices also correlate with survival in patients with breast cancer [273]. The KI-67 index, a measure of nuclear proliferation, has been shown to correlate with tumour grade, oestrogen receptor status, disease-free and overall survival in patients with breast cancer [24,231,258,327].

The precise biological mechanism activated through deregulated HGF/SF-*c-met* in breast cancer is not known. The aim of this study therefore, is to determine whether HGF/SF levels in primary breast cancer correlate with angiogenesis and tumour cell proliferation.

Materials and methods

Patients and assay for HGF/SF

The patient group and ELISA for HGF/SF are described in Chapter 3.

Assessment of tumour vascularisation (angiogenesis)

Cryostat sections of tumour were air dried and fixed in acetone. The avidin biotin-peroxidase complex staining procedure was used. Vascular endothelium was detected using a monoclonal antibody to CD-34 (QBEnd-10 clone, Serotec, Oxford, UK) used at 1/250 dilution [314,323]. In a recent report, CD34 was found to be the most strongly expressed endothelial antigen, staining more vessels reproducibly than CD31 and factor VIII [164]. Sections were incubated for 1 hour at room temperature and then washed three times with TBS. Subsequent reactions were completed using a standard DAKO StreptABComplex/HRP duet kit (Dako Ltd., Glostrup, Denmark). The peroxidase reaction was developed with diaminobenzidine-hydrogen peroxide and sections counterstained with nuclear fast red (Figure 4.1).

Counting of vessels

Intra-tumoural microvessel density was determined by a Nikon Labophot-2 microscope (Tokyo, Japan) with an ocular graticule (E11A, 19mm, Graticules Ltd., Tonbridge,

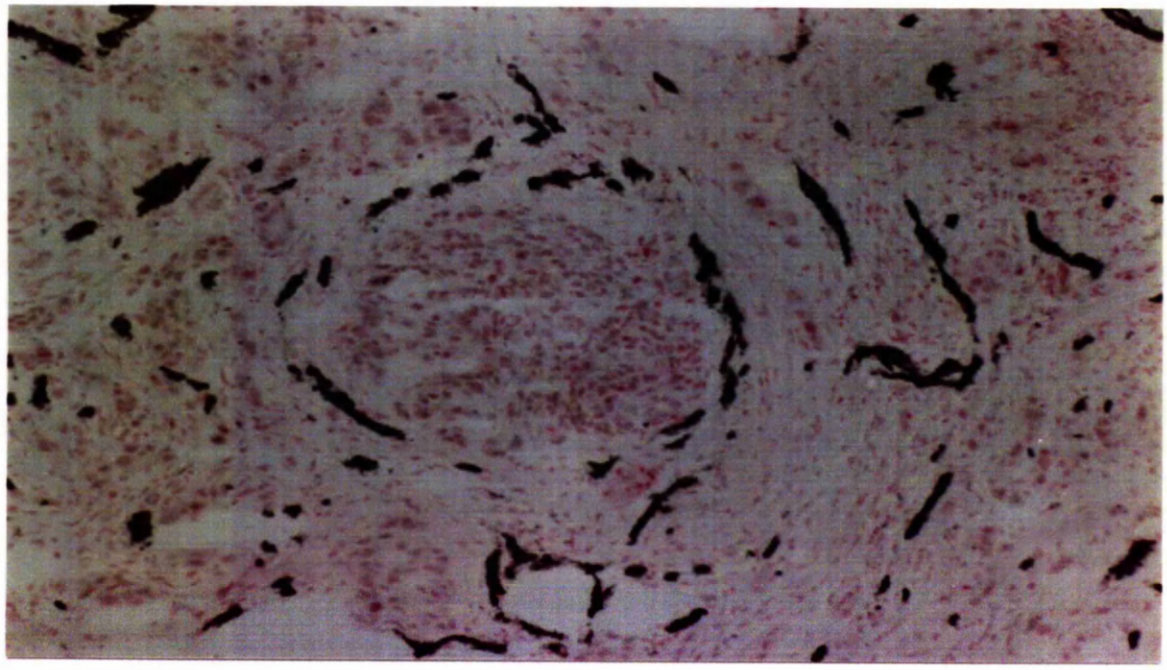


Figure 4.1

Photograph of microvessels detected by anti CD-34 antibody in a frozen section from a sample of breast cancer analysed in this study. Magnification: 100X.

Kent). Microvessel density was assessed in areas of tumour containing the most dense areas of microvessels identified under low power magnification [107,325]. A countable vessel was defined as any brown-staining endothelial cell or cluster, clearly separate from adjacent microvessels, tumour cells or connective tissue element [325]. However, the use of cryostat sections limit the spatial resolution of countable microvessels. Another method of assessing tumour angiogenesis, described by Porschen *et al* using morphometric methods for cryostat sections, was also used [228]. Tumour vascular volume was assessed using a point counting method using an eyepiece graticule. This method has been used to assess tumour angiogenesis in oesophageal and rectal cancers [227,228].

Percentage tumour vascular volume (%TVV)

The percentage tumour vascular volume was assessed using a point counting method [227]. Sections were examined at 200X magnification and a point was counted if a cross fell onto a vessel and recorded as a coincidence. A minimum of 1000 points were counted per section. The proportion of tumour occupied by blood vessels was then expressed as a ratio of coincidences/points counted x100.

Microvessel density (MVD)

Microvessels were counted from areas of maximum vessel density at 200X magnification (0.720 mm^2) [325].

Assessment of tumour proliferation

The nuclei of proliferating cells were stained with the monoclonal antibody KI-67 which detects a proliferation-associated nuclear antigen [82]. Cryostat sections air-dried and acetone-fixed were incubated for 1 hour in 1:50 dilution of MIB-1, anti-KI-67 antibody (Immunotech S.A., Marseilles, France) and washed 3 times in TBS. Subsequent reactions were completed using a standard DAKO StreptABComplex/HRP duet kit (Dako Ltd., Glostrup, Denmark). The peroxidase reaction was developed with diaminobenzidine-hydrogen peroxide and sections counterstained with nuclear fast red (Figure 4.2). Sections were counted, in tumour areas containing the highest density of stained nuclei, as determined by an initial scan at low magnification [314]. Sections were assessed at 400X magnification and nuclei scored positive if any brown staining was seen. A minimum of 1000 nuclei were counted per section. The KI-67 index was then expressed as a percentage.

Statistical analysis

The relationships between HGF/SF levels, %TVV, MVD and KI-67 index were analysed by linear regression analysis. The relationship between these variables, clinico-pathological variables, disease-free survival and overall survival was tested by Mann-Whitney or Kruskal-Wallis tests.

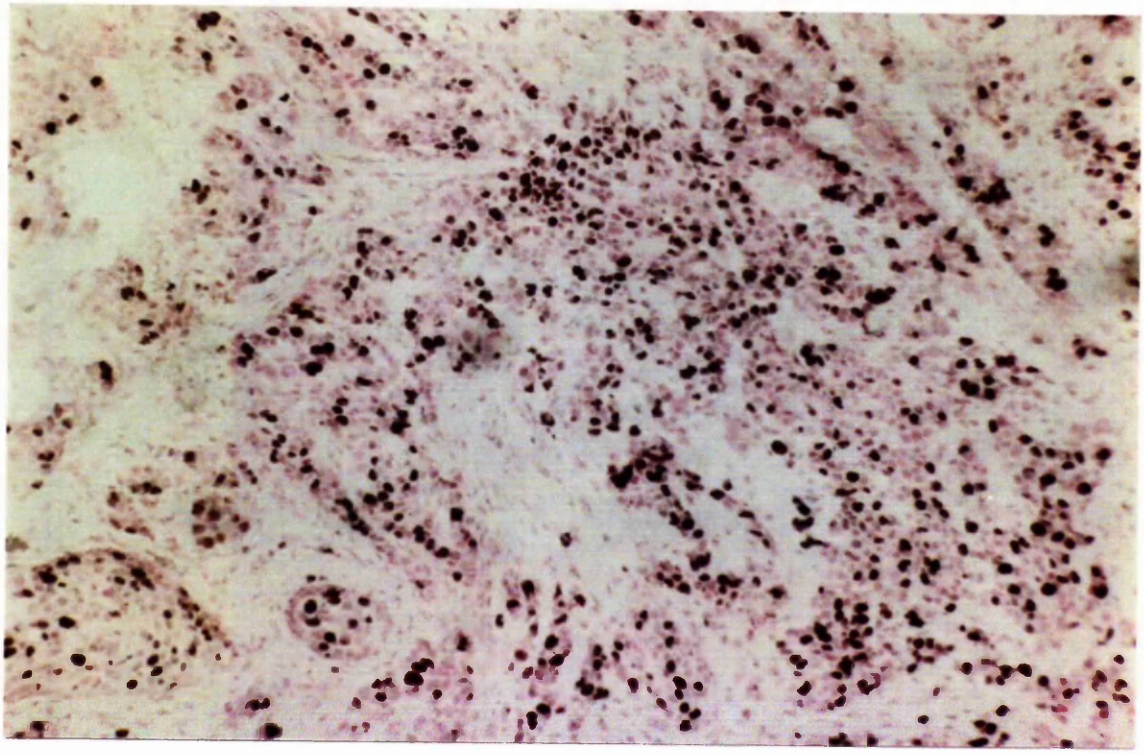


Figure 4.2

Photograph of nuclei detected by MIB-1 (KI-67) antibody in a frozen section from a sample of breast cancer analysed in this study. Magnification: 100X.

Results (appendix 3)

Patient data

The median follow-up for patients in this study was 61 months (range 7-89 months). Local recurrence was observed in 3 patients, local and systemic recurrence in 9 patients and systemic recurrence in 16 patients. All patients with systemic recurrence died from metastatic disease.

HGF/SF levels in breast cancer

The concentration of HGF/SF in breast cancers ranged between 58-1604ng/100mg total protein; mean 396ng/100mg total protein. The relationship between HGF/SF, disease-free survival, overall survival and clinico-pathological variables are discussed in Chapter 3.

Tumour vascularisation

The mean %TVV was 6.3% (median 5.7%, range 2-15%) which is similar to the mean %TVV found in rectal and oesophageal cancers by Porschen *et al* [227]. The mean MVD was 81 (median 76, range 28-229) and is similar to those reported in other studies. There was a significant correlation between %TVV and MVD, $r=0.648$, $p<0.001$ (Figure 4.3). Tumour levels of HGF/SF demonstrated a significant positive linear

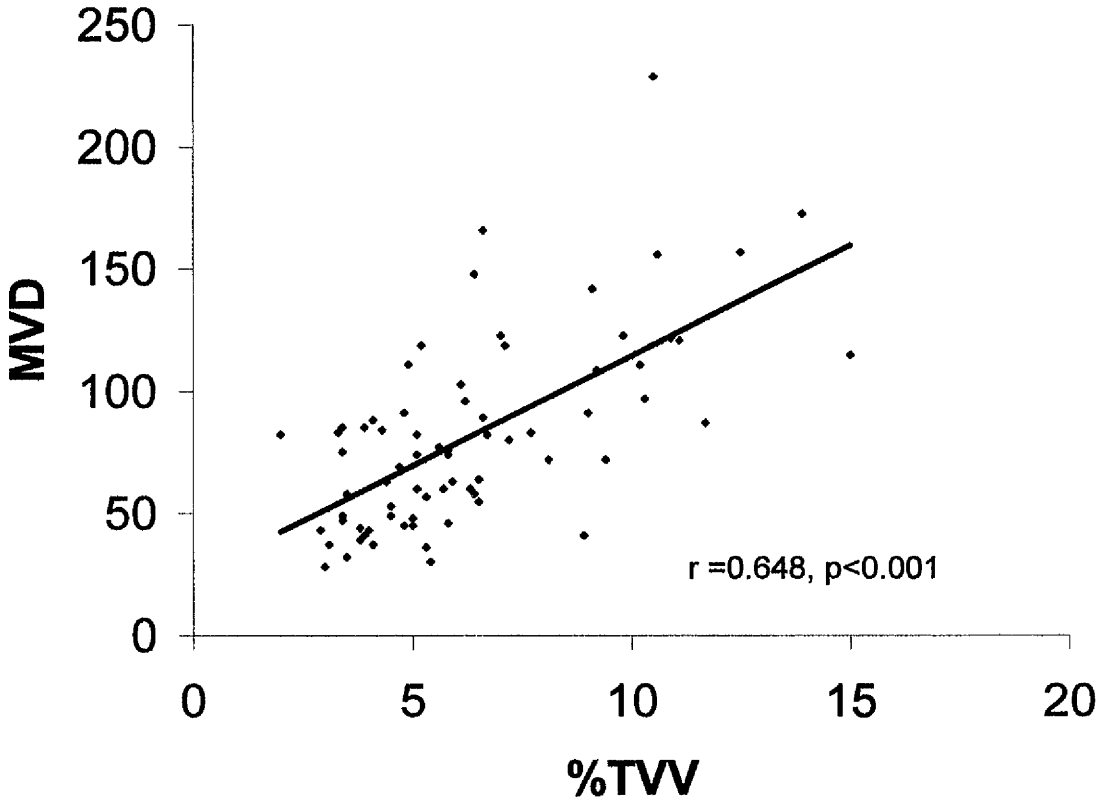


Figure 4.3

Scatterplot showing relationship between tumour microvessel density (MVD) and percentage tumour vascular volume (%TVV).

relationship with %TVV: r=0.493, p<0.001, (Figure 4.4) and MVD: r=0.279, p<0.02, (Figure 4.5, Table 4.1). In addition, %TVV correlated significantly with reduced disease-free survival, p<0.04, (Table 4.1). There were no significant correlations between %TVV or MVD, overall survival and clinico-pathological prognostic variables (Table 4.1 and 4.2).

Tumour proliferation

The mean KI-67 index was 19.9% (median 19%, range 4-59%). There was a significant positive linear correlation between HGF/SF levels and KI-67 index: r=0.255, p<0.04, (Figure 4.6). KI-67 index correlated significantly with overall survival, p<0.05, (Table 4.3). There was a non-significant trend between histological grade and KI-67 index. However, when KI-67 index in well differentiated breast cancers (grade 1, median 12.0) was compared to moderate-poorly differentiated cancers (grades 2 and 3, median 20.0) this trend reached significance, p<0.02, Mann-Whitney. There were no significant correlation between KI-67 index, %TVV, disease-free survival and clinico-pathological prognostic variables (Table 4.3).

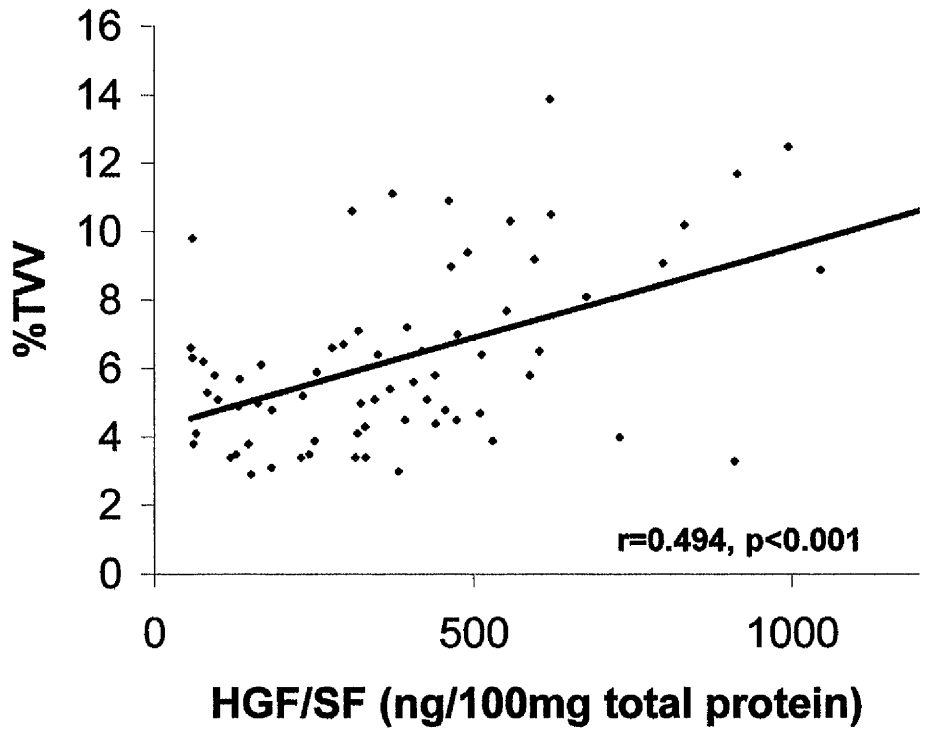


Figure 4.4

Scatterplot showing relationship between percentage tumour vascular volume (%TVV) and HGF/SF levels in breast cancer patients.

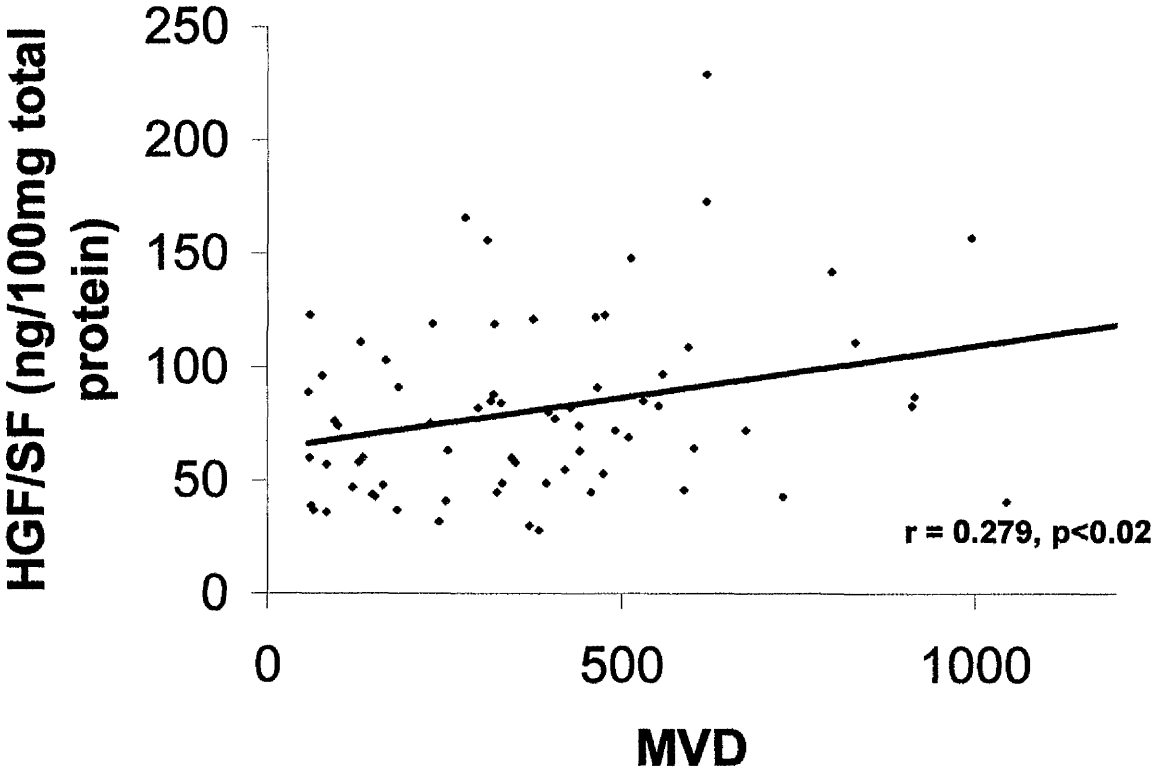


Figure 4.5

Scatterplot demonstrating relationship between microvessel density (MVD) to HGF/SF levels in human breast cancer patients.

Table 4.1

Univariate analysis of the relationship between % tumour vascular volume (%TVV) to prognostic variables. The relationship between HGF/SF and tumour size to %TVV was tested by linear regression analysis. The relationship between disease-free survival, overall survival, nodal status, tumour grade, oestrogen receptor and vascular invasion to %TVV was tested by Mann-Whitney test. The relationship between tumour grade and %TVV was tested by Kruskal-Wallis test.

Parameter	**n**	**Correlation to %TVV**	**p value**
HGF/SF	71	R=0.493	<0.001*
Tumour size	72	R=0.197	0.1

Parameter	**Values**	**n**	**Median %TVV**	**p value**
Disease-free survival	Relapse	27	6.5	0.04*
	Disease-free	45	5.3	
Overall Survival	Dead	24	6.4	0.21
	Alive	48	5.35	
Nodal status	Positive	34	5.85	0.19
	Negative	37	5.1	
Histological grade	1	12	5.05	0.34
	2	31	5.8	
	3	26	5.7	
Oestrogen receptor	Positive	29	5.1	0.66
	Negative	31	5.6	
Lymphatic invasion	Positive	21	6.1	0.6
	Negative	51	5.3	

* statistically significant

% TVV not available in one patient. HGF/SF levels not available in 2 patients.

Lymph node status unknown in 4 patients.

Tumours not graded in 7 patients.

Oestrogen receptor status unknown in 12 patients.

Table 4.2

Univariate analysis of the relationship between tumour microvessel density (MVD) to prognostic variables. The relationship between HGF/SF and tumour size to MVD was tested by linear regression analysis. The relationship between disease-free survival, overall survival, nodal status, oestrogen receptor and vascular invasion to MVD was tested by Mann-Whitney test. The relationship between tumour grade and MVD was tested by Kruskal-Wallis test.

Parameter	**n**	**Correlation to MVD**	**p value**
HGF/SF	71	0.279	0.019*
Tumour size	72	0.147	0.217

Parameter	**Values**	**n**	**Median MVD**	**p value**
Disease-free survival	Relapse	27	83.0	0.11
	Disease-free	45	69.0	
Overall Survival	Dead	24	82.0	0.48
	Alive	28	74.5	
Nodal status	Positive	33	82.0	0.45
	Negative	36	74.0	
Histological grade	1	8	55.0	0.244
	2	31	84.0	
	3	26	78.5	
Oestrogen receptor	Positive	30	74.0	0.92
	Negative	31	72.0	
Lymphatic invasion	Positive	21	83.0	0.68
	Negative	51	75.0	

* statistically significant

% TVV not available in one patient. HGF/SF levels not available in 2 patients.

Lymph node status unknown in 4 patients.

Tumours not graded in 7 patients.

Oestrogen receptor status unknown in 12 patients.

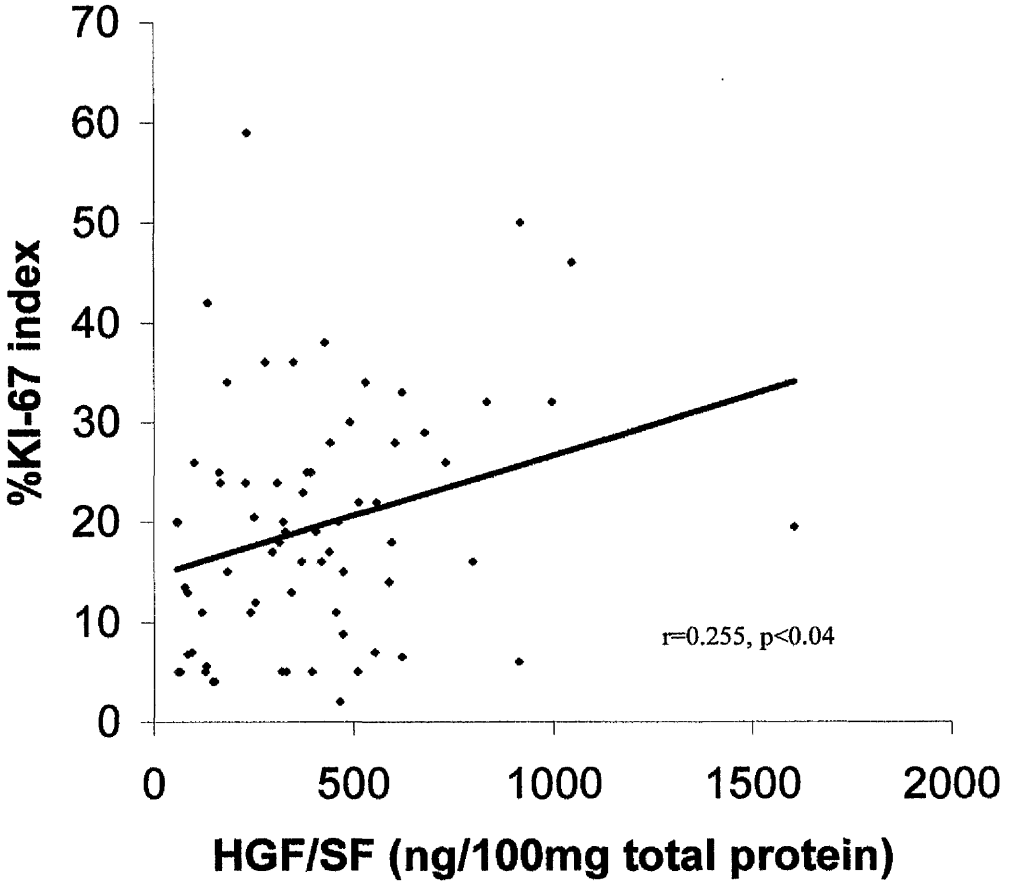


Figure 4.6

Scatterplot demonstrating relationship between KI-67 index and HGF/SF levels in breast cancer patients.

Table 4.3

Univariate analysis of the relationship between KI-67 index and prognostic variables. The relationship between HGF/SF, %TVV and tumour size to KI-67 was tested by linear regression analysis. The relationship between disease-free survival, overall survival, nodal status, oestrogen receptor and vascular invasion to KI-67 was tested by Mann-Whitney test. The relationship between tumour grade and KI-67 was tested by Kruskal-Wallis test.

Parameter	n	Correlation to KI-67	p value
HGF/SF	71	0.255	<0.04*
%TVV	72	0.288	0.56
Tumour size	72	-0.022	0.97

Parameter	Values	n	Median KI-67	p value
Disease-free survival	Relapse	28	21.5	0.15
	Disease-free	44	16.5	
Overall Survival	Dead	24	25.0	0.046*
	Alive	48	16.5	
Nodal status	Positive	35	19.0	0.87
	Negative	37	20.0	
Histological grade	1	8	10.9	0.081
	2	31	22.0	
	3	26	19.25	
Oestrogen receptor	Positive	30	16.5	0.19
	Negative	31	19.0	
Lymphatic invasion	Positive	21	20.0	0.47
	Negative	50	19.0	

* statistically significant

% TVV not available in one patient. HGF/SF levels not available in 2 patients.

Lymph node status unknown in 4 patients.

Tumours not graded in 7 patients.

Oestrogen receptor status unknown in 12 patients.

Discussion

Tumour proliferation and angiogenesis are important prognostic variables in breast cancer. Many of these functions are controlled by growth factors through their receptors via autocrine or paracrine loops. However, the intrinsic and extrinsic factors that control these variables are the subject of ongoing investigation. Tumour growth and metastasis are dependent on angiogenesis [323]. Studies have demonstrated a significant relationship between angiogenesis and metastasis in patients with breast cancer [80,107,325]. The development of angiogenesis requires a complex cascade of events controlled by soluble or tissue-bound factors [139]. Factors such as VEGF and basic FGF are thought to play an important part in breast cancer angiogenesis and growth [2,298,299]. VEGF, produced by normal and malignant epithelial cells, is a paracrine mediator of endothelial cell growth binding to receptors Flt-1 (VEGF receptor-1) and KDR (VEGF receptor-2) [163]. Several studies have found an association between VEGF expression and relapse-free and overall survival, with significant shorter survival times in VEGF-rich tumours [79,299]. In addition factors such as basic FGF, TGF-α, TGF-β and EGF receptor expression has been shown to correlate with microvessel density in human breast cancer [50]. In particular TGF-α/EGF-receptor paracrine and TGFβ/TGFβ-receptor autocrine endothelial loops correlate significantly with tumour microvessel density [50].

HGF/SF has been shown to possess potent angiogenic properties both *in vitro* and *in vivo*. Both large vessel and microvessel endothelial cells express the *c-met* receptor and are biologically responsive to HGF/SF [25,94,192,243,245,249]. HGF/SF has been shown to

promote proliferation, motility, invasion and capillary morphogenesis of endothelial cells *in vitro*. In addition to producing HGF/SF, vascular smooth muscle cells express *c-met* receptor and are responsive to HGF/SF [94,126,129,249]. *In vitro* studies suggest that HGF/SF may interact with other cytokines, such as TNF-α and IL-1 in promoting tumour invasion and angiogenesis [251]. HGF/SF has been shown to stimulate angiogenesis *in vivo* using the mouse matrigel assay and a rat cornea assay [94,192]. In both reports, HGF/SF induced angiogenesis in a dose-dependent fashion with a maximal response similar in intensity to those induced by basic FGF or VEGF. Histological analysis of the assays excluded the possibility of HGF/SF stimulating angiogenesis through inflammation.

This study has demonstrated a correlation between tumour levels of HGF/SF and indices of tumour neovascularisation suggesting that HGF/SF may be linked to tumour angiogenesis in breast cancer. This observation has been subsequently confirmed [335,147]. HGF/SF levels have been shown to be significantly associated with tumour levels of von Willebrand's factor (vWF), an endothelial-specific marker protein, in patients with breast cancer [335]. Transfection of HGF/SF into the human breast cancer cell line MDA-MB-231 results in tumours growing more rapidly than non-transfected cells when implanted into mammary pads of nude mice [147]. The proliferation rates *in vitro* of HGF/SF transfected MDA-MB-231 breast cancer cells however was no greater than in non-transfected cells. Histological analysis of the tumours produced by HGF/SF transfected cells demonstrated significantly higher microvessel densities when compared to tumours derived from non-transfected cells. Increased tumour growth rate

in this model appeared to be due, in part, to an angiogenic response induced by HGF/SF. Laterra *et al* observed higher growth rates and microvessel densities in HGF/SF transfected 9L rat glioma cells implanted into rats when compared to non-transfected tumour cells [149]. Similar observations have also been reported in HGF/SF transfected human glioblastoma cells grown in immunocompromised mice [150]. These studies confirm the observations of the present study and further support the hypothesis of linking HGF/SF and tumour angiogenesis. In contrast, in a series of 135 primary breast cancers Toi *et al* were unable to demonstrate a correlation between HGF/SF, VEGF or basic FGF levels with tumour microvascular density [300]. Thus, further studies are necessary to confirm the observed correlation between HGF/SF and tumour angiogenesis in human breast cancer. This is important because angiogenesis is an early event in tumour development and is essential for tumour growth, metastasis and survival of metastatic deposits. Truncated peptide fragments of the α-chain of HGF/SF, designated HGF/SF NK-1 and NK-2, have been shown to antagonise HGF/SF-mediated angiogenesis [248]. These and other HGF/SF antagonists may provide new therapeutic strategies for the treatment of breast cancer.

Proliferation is an important prognostic variable in breast cancer. Many studies have shown that a high number of mitotic figures, high KI-67 index, high tritiated thymidine or bromodeoxyuridine labeling indices and a high percentage S-phase are associated with poor prognosis in breast cancer [36]. The KI-67 index has been shown to correlate with tumour grade, oestrogen receptor status and overall survival in breast cancer [24,113,231,258,327]. Similarly, in this study KI-67 index correlated reduced overall survival in

patients with breast cancer. Tumour cell proliferation may be controlled by various growth factors interacting via autocrine or paracrine loops. A number of growth factors and their receptors have been shown both *in vitro* and *in vivo* to relate to tumour cell proliferation in breast cancer. The type 1 family of growth factor receptors, which include EGF receptor and c-erbB-2 are expressed in both normal breast ductal epithelium and in some breast cancers [97]. Growth of normal human mammary epithelial cells is dependent on the synergistic interaction between EGF and IGF-1 when grown in serum-free conditions [65]. The receptor to EGF and its physiological ligands EGF and TGF-α are overexpressed in around 50% of primary human breast cancers [302]. High levels of EGF receptor have been shown to correlate with poor prognosis and failure to respond to hormone therapy in breast cancer [97,99,207]. *In vitro*, autocrine growth dependence on the TGF-α/EGF-receptor system has been observed in the MDA MB-468 breast cancer cell line [64]. Increased EGF receptor expression has been shown to correlate with indices of tumour proliferation in patients with breast cancer [50,111,211]. Furthermore, De Jong *et al* observed that the combined expression of TGF-α/EGF-receptor demonstrated a stronger correlation to mitotic activity than EGF receptor expression alone [50]. The epithelial tyrosine kinase receptor encoded by c-erbB-2 is overexpressed at very high levels in about 20% of breast cancers and is indicative of poor prognosis [97]. The heregulins are a family of growth factors that activate erbB-2 when present as a heterodimer with erbB-3, EGF receptor and erbB-4 and have been shown to support growth of human mammary epithelial cells in serum-free conditions in the absence of EGF and IGF-1 [232,330]. Studies *in vitro* demonstrate that constitutive autocrine activation of c-erbB-2 receptor in MCF-7 breast cancer cells transfected with

heregulin results in enhanced proliferation [160]. In addition, c-erbB-2 overexpression in human breast epithelial cells has been shown to confer growth factor independence [330]. Tumour proliferation indices *in vivo* have been shown to correlate with c-erbB-2 expression in human breast cancers [111]. TGF-β is the most potent known inhibitor of the progression of normal mammary epithelial cells through the cell cycle [236]. It is expressed in both normal mammary epithelium and stromal cells and may be involved in both autocrine and paracrine growth inhibition [175,237]. Although most breast cancer cell lines are growth inhibited by TGF-β, there is little evidence that this occurs *in vivo* [50,302]. Indeed, Auvinen *et al* found a negative correlation between the S-phase fraction and TGF-β [4]. However, Dublin *et al* found no correlation between these variables in breast cancer and similarly, De Jong *et al* failed to demonstrate any relationship between TGF-β expression and mitotic activity [50,60]. Paradoxically, increased expression of TGF-β has also been found to correlate with poor prognostic features in breast cancer [316]. Thus in breast cancer the negative autocrine effect of TGF-β on breast epithelial cells may be lost, although the clinical significance of these observations is as yet uncertain.

In vitro studies have shown that HGF/SF induces growth in a variety of benign and malignant epithelial cell types including breast [115,252]. The results of this study suggest a link between HGF/SF levels and tumour proliferation *in vivo*. However, this observation has not been confirmed. Animal experiments have shown that the rate of tumour cell proliferation is inversely proportional to the tumour microvessel distance [296]. Tumour vascularisation may therefore influence tumour cell proliferation and in the

present study there was a non-significant trend between tumour vascular volume and KI-67 index (p=0.056). Lamszus *et al* reported that enhanced tumour proliferation induced by HGF/SF in breast cancer cells was a function of angiogenesis rather than direct stimulation of cell growth [147]. In support of this observation suppression of VEGF induced angiogenesis has been shown to suppress tumour growth *in vivo* [136].

In conclusion, this study demonstrates a significant correlation between HGF/SF levels, % tumour vascular volume (TVV), microvessel density (MVD) and KI-67 index. These results provide further supportive evidence that HGF/SF may be linked to the mechanisms of tumour progression.

Chapter 5: Conclusions

HGF/SF-*c-met* is involved in stromal-epithelial regulation of cellular processes such as growth, dissociation, motility and invasiveness [6,313]. When coordinated they appear to play an important role in morphogenesis of epithelial tissues [19]. There is increasing evidence that HGF/SF-*c-met* is an essential regulating component of breast duct development [13,208,276,334]. The results of this thesis support the hypothesis that aberrant HGF/SF-*c-met* expression occurs in human breast cancer and is related to disease progression.

Investigation on frequency of loss of heterozygosity of the *c-met* proto-oncogene in human breast cancer

Aberrant HGF/SF-*c-met* function in breast cancer was first suggested by the observation of a high frequency of LOH at 7q31, the *c-met* locus, in human breast cancer patients [16]. Furthermore, LOH of this gene was related to a significant reduction in both disease-free and overall survival. This finding suggested the presence of an important tumour suppressor gene at 7q31, possibly *c-met*, in human breast cancer. In our series of 111 patients with primary breast cancer, 52 were heterozygous and LOH at 7q31 was observed in only 4% of these cases. The presence of a tumour suppressor gene at 7q31 is controversial and the identity of this putative gene is unknown. In human breast cancer the reported frequency of LOH at this locus ranges between 4-83%. However, in a series of 683 breast cancers, Devilee *et al* observed LOH at 7q31 in 19% of cases with

no prognostic significance [52]. Furthermore, a recent study by Bieche's group observed LOH at 7q31 in 20% of breast cancers [59]. The wide range of frequencies of LOH at 7q31 may be due to a fragile site, FRA7G, located at 7q31.1 [116]. Loss of heterozygosity may therefore be the result of intrinsic chromosome instability rather than the presence of a tumour suppressor gene. The identity of any putative tumour suppressor gene on 7q remains unknown. Microsatellite studies of 7q31.1 have identified several areas of frequent deletion, (D7S522, D7S480, D7S486 and D7S480-D7S650), which do not include the *c-met* locus [17,116,342]. Furthermore, the biological properties of *c-met* are clearly those of a proto-oncogene.

HGF/SF-*c-met* ligand receptor system in human breast cancer

The work presented in this thesis has demonstrated a statistical association between HGF/SF and *c-met* in relation to disease-relapse in patients with primary breast cancer. This observation provides strong support to the hypothesis that aberrant HGF/SF-*c-met* expression occurs in breast cancer. Furthermore, the data confirm the presence of increased HGF/SF levels in human breast cancer when compared to tumour-free breast tissue. HGF/SF levels demonstrated significant associations with disease-relapse and death. However, HGF/SF levels did not correlate with other clinico-pathological variables. These findings are concordant with three similar studies [331,332,335]. *C-met* protein was observed in all samples of tumour-free breast tissue and in a proportion of patients with breast cancer. Patients with detectable *c-met* demonstrated a significant association with disease-relapse. This pattern of receptor expression and relationship to

disease progression has subsequently been confirmed [86].

Although HGF/SF-*c-met* appears to be an important paracrine regulator of breast duct development, several in-situ hybridisation studies have detected HGF/SF mRNA in both benign and malignant breast epithelial cells [13,126,208,276,307,317,334]. This observation suggests that both autocrine and paracrine signalling are possible with HGF/SF-*c-met*. Autocrine HGF/SF-*c-met* activation has been shown to induce tumorigenesis, invasion and metastasis in breast epithelial cells both *in vitro* and *in vivo* [115,176,290]. HGF/SF-*c-met* expression is regulated both systemically and locally. Oestrogen and progesterone are able to upregulate both HGF/SF and *c-met* expression [157,186]. At the cellular level, breast epithelial cells regulate fibroblast production of HGF/SF through SF-IF (positive) and TGF-β (negative) pathways [246,267,268]. Increased levels of HGF/SF also down-regulate *c-met* expression [240]. It is possible that in the majority of breast cancers increased HGF/SF levels down-regulate *c-met* expression through autocrine or paracrine signalling. Failure of regulatory mechanisms may allow inappropriate *c-met* expression with uncontrolled growth, dissociation, invasion and ultimately metastasis. This failure may involve the formation of an HGF/SF-*c-met* autocrine loop.

HGF/SF, angiogenesis and tumour cell proliferation in primary breast cancer

A significant correlation between HGF/SF levels and tumour angiogenesis was observed in patients with breast cancer. HGF/SF has been shown to promote angiogenesis both *in vitro* and *in vivo* and formation of a HGF/SF-*c-met* autocrine loop

in breast cancer cells will promote tumour angiogenesis in mice [147,248]. Similar studies have confirmed that HGF/SF promotes tumour angiogenesis in other tumour types *in vivo* [149,150]. Further evidence of a link between HGF/SF and tumour angiogenesis is provided by the observation of a direct relationship between HGF/SF and von Willebrand's factor in breast cancer [335]. However, in a study of 135 patients with breast cancer, Toi *et al* failed to demonstrate any relationship between HGF/SF and tumour microvessel density [300]. In this study, HGF/SF levels significantly correlated with the KI-67 index and therefore with tumour proliferation. HGF/SF is known to promote breast epithelial cell growth *in vitro* [115,208,252]. However, HGF/SF promotion of breast cancer *in vivo* appears to be related to angiogenesis rather than direct stimulation of cellular proliferation [147]. The observations of the present study regarding HGF/SF and tumour proliferation in breast cancer thus remain unconfirmed.

HGF/SF as a possible target in cancer treatment

The metastatic spread of tumours is the most important factor in determining survival in breast cancer patients. The current knowledge of the effects of HGF/SF mediated by *c-met* on tumour cells both *in vitro* and *in vivo* imply that it may play a role in tumour dissemination *in vivo*. HGF/SF thus presents a possible target for anti-tumour therapy. Several factors are known to inhibit the action of HGF/SF. Gamma-linolenic acid (GLA) has been shown to be cytotoxic toward cancers both *in vitro* and *in vivo* [117]. GLA has been shown to inhibit HGF/SF stimulated tumour cell motility, invasion and membrane ruffling at non-toxic cellular concentrations possibly through the regulation

of E-cadherin [119,120,121]. A naturally occurring HGF/SF antagonist that specifically inhibits HGF/SF induced mitogenesis has been identified [31]. This antagonist is an alternative HGF/SF transcript that may compete with binding to the *c-met* receptor. The N-terminal hairpin domain truncated transcripts also possess antagonistic behavior. HGF/SF NK-1 and NK-2 are naturally occurring antagonists of HGF/SF-induced matrix degradation [61]. However, HGF/SF NK-1, NK-2 and NK-3 all stimulate motility [35,100,112]. HGF/SF NK-4 completely inhibits HGF/SF-stimulated mitogenic, motogenic and morphogenic activities [45]. Furthermore, NK-4 possesses no other biological activity and may be a candidate for future therapeutic investigation. HGF/SF-induced tumour cell motility and invasion are also inhibited by both interleukins 4 and 12 [103,106,308]. HGF/SF induced cancer cell invasion can be inhibited by increasing E-cadherin expression [120,121]. Restoring E-cadherin cell-cell adhesion by Tiam 1 or RacV12 reduces HGF/SF induced cell dissociation [108]. Thus several mechanisms have been identified *in vitro* that may form the basis of direct cellular antagonism of the effects of HGF/SF on tumour *in vivo*.

HGF/SF action may be inhibited through action on pro-HGF/SF cleavage mechanisms. In response to tissue injury, HGF activator (HGFA) is activated and cleaves pro-HGF/SF. Two inhibitors of HGFA have recently been identified and are produced by the MKN45 gastric carcinoma cell line [132,272]. Regulation of HGFA expression through HGFA inhibitors may represent another pathway of modulating HGF/SF effects in tumour.

In conclusion, HGF/SF-*c-met* activity in tumour may play an important role in disease progression. Inhibition of this interaction by agents such as GLA, HGF/SF NK-4 or interleukins 4 and 12 may provide new anti-metastatic treatment. However, the effects of these inhibitors have only been studied *in vitro*. Further investigations are necessary on the effects, efficacy and safety of these antagonists *in vivo*.

References

1. Aggeler, J., Ward, J., Blackie, L.M., Barcellos Hoff, M.H., Streuli, C.H., Bissell, M.J. (1991) Cytodifferentiation of mouse mammary epithelial cells cultured on a reconstituted basement membrane reveals striking similarities to development in vivo. Journal of Cell Science. 99. 407-417.

2. Anandappa, S.Y., Winstanley, J.H.R., Leinster, S., Green, B., Rundland, P.S., Barraclough, R. (1994) Comparative expression of fibroblast growth factor mRNA in benign and malignant breast disease. British Journal of Cancer. 69. 772-776.

3. Aravamudan, B., Watabe, M., Watabe, K. (1993) Characterization of the 5'-flanking region of the hepatocyte growth factor gene. Biochemical and Biophysical Research Communications. 195. 346-353.

4. Auvinen, P., Lipponen, P., Johansson, R., Syrjanen, K. (1995) Prognostic significance of TGF-β1 and TGF-β2 expression in human breast cancer. Anticancer Research. 15. 2627-2632.

5. Barcellos Hoff, M.H., Aggeler, J., Bissell, M.J. (1989) Functional differentiation and alveolar morphogenesis of primary mammary cultures on reconstituted basement membrane. Development. 105. 223-235.

6. Bardelli, A., Comoglio, P.M. (1997) Scatter factor receptors are key players in a unique multistep program leading to invasive growth. Ciba Foundation Symposium. 212. 133-147.

7. Bardelli, A., Longati, P., Albero, D., Goruppi, S., Sneider, C., Ponzetto, C., Comoglio, PM. (1996) HGF receptor associates with the anti-apoptotic protein BAG-1 and prevents cell death. EMBO Journal. 15. 6205-6212.

8. Bardelli, A., Maina, F., Gout, I., Fry, M.J., Waterfield, M.D., Comoglio, P.M., Ponzetto, C. (1992) Autophosphorylation promotes complex formation of recombinant hepatocyte growth factor receptor with cytoplasmic effectors containing SH2 domains. Oncogene. 7. 1973-1978.

9. Belfiore, A., Gangemi, P., Costantino, A., Russo, G., Santonocito, GM., Ippolito, O., Di Renzo, MF., Comoglio, P., Fiumara, A., Vigneri, R. (1997) Negative/low expression of the Met/HGF receptor identifies papillary thyroid carcinomas with high risk of distant metastases. Journal of Clinical Endocrinology and Metabolism. 82. 2322-2328.

10. Bellusci, S., Moens, G., Gaudino, G., Comoglio, P., Nakamura, T., Thiery, J.P., Jouanneau, J. (1994) Creation of an hepatocyte growth factor/scatter factor autocrine

loop in carcinoma cells induces invasive properties associated with increased tumorigenicity. Oncogene. 9. 1091-1099.

11. Bennett, D.C. (1980) Morphogenesis of branching tubules in cultures of cloned mammary epithelial cells. Nature. 285. 657-659.

12. Bennett, J.H., Furness, J., Atkin, P., Speight, P.M. (1997) Scatter factor (SF) regulation of matrix metalloproteinase production by oral carcinoma cells. Journal of Dental Research. 76. 2140.

13. Berdichevsky, F., Alford, D., D'Souza, B., Taylor-Papadimitriou, J. (1994) Branching morphogenesis of human mammary cells in collagen gels. Journal of Cell Science. 107. 3557-3568.

14. Besser, D., Bardelli, A., Didichenko, S., Thelen, M., Comoglio, PM., Ponzetto, C., Nagamine, Y. (1997) Regulation of the urokinase-type plasminogen activator gene by the oncogene Tpr-Met involves GRB2. Oncogene. 14. 705-711.

15. Bhargava, M., Joseph, A., Knesel, J., Halaban, R., Li, Y., Pang, S., Goldberg, I., Setter, E., Donovan, M.A., Zarnegar, R., Michalopoulos, G.A., Nakamura, T., Faletto, D., Rosen, E.M. (1992) Scatter factor and hepatocyte growth factor: activities, properties and mechanism. Cell Growth and Differentiation. 3. 11-20.

16. Bieche, I., Champeme, M.H., Matifas, F., Hacene, K., Callahan, R., Lidereau, R. (1992) Loss of heterozygosity on chromosome 7q and aggressive primary breast cancer. Lancet. 339. 139-143.

17. Bieche, I., Khodja, A., Driouch, K., Lidereau, R. (1997) Genetic alteration mapping on chromosome 7 in primary breast cancer. Clinical Cancer Research. 3. 1009-1016.

18. Birchmeier, C., Birchmeier, W. (1993) Molecular aspects of mesenchymal-epithelial interactions. Annual reviews of cell biology. 9. 511-540.

19. Birchmeier, W., Brinkmann, V., Niemann, C., Meiners, S., DiCesare, S., Naundorf, H., Sachs, M. (1997) Role of HGF/SF and *c-met* in morphogenesis and metastasis of epithelial cells. Ciba Foundation Symposium. 212. 230-246.

20. Bladt, F., Riethmacher, D., Isenmann, S., Aguzzi, A., Birchmeier, C. (1995) Essential role for the *c-met* receptor in the migration of myogenic precursor cells into the limb bud. Nature. 376. 768-771.

21. Boccaccio, C., Gaudino, G., Gambarotta, G., Galimi, F., Comoglio, P.M. (1994) Hepatocyte growth factor (HGF) receptor expression is inducible and is part of the delayed-early response to HGF. Journal of Biological Chemistry. 269. 12846-12851.

22. Borset, M., Lien, E., Espevik, T., Helseth, E., Waage, A., Sundan, A. (1996) Concomitant expression of hepatocyte growth factor/scatter factor and the receptor *c-met* in human myeloma cell-lines. Journal of Biological Chemistry. 271. 24655-24661.

23. Bottaro, D.P., Rubin, J.S., Faletto, D.L., Chan, A.M.L., Kmiecik, T.E., Vande Woude, G.F., Aaronson, S.A. (1991) Identification of the hepatocyte growth factor receptor as the *c-met* proto-oncogene product. Science. 251. 802-804.

24. Bouzubar, N., Walker, K.J., Griffiths, K., Ellis, IO., Elston, CW., Robertson, JFR, Blamey, RW. (1989) Ki 67 immunostaining in primary breast cancer:pathological and clinical associations. British Journal of Cancer. 59. 943-947.

25. Bussolino, F., Di Renzo, M.F., Ziche, M., Bocchietto, E., Olivero, M., Naldini, L., Gaudino, G., Tamagnone, L., Coffer, A., Comoglio, P.M. (1992) Hepatocyte growth factor is a potent angiogenic factor which stimulates endothelial cell motility and growth. Journal of Cell Biology. 119. 629-641.

26. Byers, S., Park, M., Sommers, C., Seslar, S. (1994) Breast carcinoma: A collective disorder. The Breast. 31. 203-215.

27. Cairns, P., Polascik, T.J., Eby, Y., Tokino, K., Califano, J., Merlo, A., Mao, L., Herath, J., Jenkins, R., Westra, W., Rutter, J.L., Buckler, A., Gabrielson, E., Tockman, M., Cho, K.R., Hedrick, L., Bova, G.S., Isaacs, W., Koch, W., Schwab, D., Sidransky, D. (1995) Frequency of homozygous deletion at p16/CDKN2 in primary human tumours. Nature Genetics. 11. 210-212.

28. Callahan, R., Cropp, C., Sheng, Z.M., Merlo, G., Steeg, P., Liscia, D., Lidereau, R. (1993) Definition of regions of the human genome affected by loss of heterozygosity in primary human breast tumours. Journal of Biological Chemistry. S17G. 167-172.

29. Camps, J.L., Chang, S., Hsu, T.C., Freeman, MR., Hong, SJ., Zhau, HE., Von Eschenbach, AC., Chung, LWK. (1990) Fibroblast-mediated acceleration of human epithelial tumour growth in vivo. Proceedings of the National Academy of Sciences of the United States of America. 87. 75-79.

30. Carter, S.L., Negrini, M., Baffa, R., Gillum, D.R., Rosenberg, A.L., Schwartz, G.F., Croce, C.M. (1994) Loss of heterozygosity at 11q22-q23 in breast cancer. Cancer Research. 54. 6270-6274.

31. Chan, A.M.L., Rubin, J.S., Bottaro, D.P., Hirschfield, D.W., Chedid, M., Aaronson, S.A. (1991) Identification of a competitive HGF antagonist encoded by an alternative transcript. Science. 254. 1382-1385.

32. Chen, L.C., Kurisu, W., Ljung, B.M., Goldman, E.S., Moore, D., Smith, H.S. (1992) Heterogeneity for allelic loss in human breast cancer. Journal of the National Cancer Institute. 84. 506-510.

33. Chen, L.C., Matsumura, K., Deng, G., Kurisu, W., Ljung, B.M., Lerman, M.I., Waldmann, F.M., Smith, H.S. (1994) Deletion of two seperate regions on chromosome 3 in breast cancers. Cancer Research. 54. 3021-3024.

34. Chen, P., Murphy-Ullrich, J.E., Wells, A. (1996) A role for gelsolin in actuating epidermal growth factor receptor-mediated cell motility. Journal of Cell Biology. 134. 689-698.

35. Cioce, V., Csaky, K.G., Chan, A.M.L., Bottaro, DP., Taylor, WG., Jensen, R., Aaronson, SA., Rubin, JS. (1996) HGF/NK-1 is a naturally occuring HGF/SF variant with partial agonist/antagonist activity. Journal of Biological Chemistry. 271. 13110-13115.

36. Clark, G.M. (1996). Prognostic and predictive factors. Diseases of the Breast. Harris, J.R., Lippman, M.E., Morrow, M., Hellman, S. 461-485. Philadelphia: Lippincott-Raven Publishers.

37. Clarke, R, Dickson, R.B., Lippman, M.E. (1992) Hormonal aspects of breast cancer. Growth factors, drugs and stromal interactions. Critical Reviews in Oncology and Hematology. 12. 1-23.

38. Collins, N., McManus, R., Wooster, R., Mangion, J., Seal, S., Lakhani, S.R., Ormiston, W., Daly, P.A., Ford, D., Easton, D.F., Straton, M.R. (1995) Consistent loss of wild type allele in breast cancers from a family linked to the BRCA2 gene on chromosome 13q12-13. Oncogene. 10. 1673-1675.

39. Cooper, C.S., Park, M., Blair, D.G., Tainsky, M.A., Huebner, K., Croce, C.M., Vande Woude, G.F. (1984) Molecular cloning of a new transforming gene from a chemically transformed human cell line. Nature. 311. 29-33.

40. Cooper, C.S., Tempest, P.R., Beckman, P.M., Heldin, C.H., Breakers, P. (1986) Amplification and overexpression of met gene in spontaneously transformed NIH/3T3 fibroblast. EMBO Journal. 5. 2623-2628.

41. Cornelisse, C.J. (1992) Loss of heterozygosity, chromosome 7q, and breast cancer(Letter). Lancet. 339. 1423.

42. Corps, A.N., Sowter, H.M., Smith, S.K. (1997) HGF stimulates motility, chemotaxis and mitogenesis in ovarian carcinoma cells expressing high levels of *c-met*. International Journal of Cancer. 73. 151-155.

43. Crepaldi, T., Pollack, A.L., Prat, M., Zborek, A., Mostov, K., Comoglio, P.M. (1994) Targeting of the SF/HGF receptor to the basolateral domain of polarized epithelial cells. Journal of Cell Biology. 125. 313-320.

44. Danielson, K.G., Oborn, C.J., Durban, E.M., Butel, J.S., Medina, D. (1984) Epithelial mammary cell line exhibiting normal morphogenesis in vivo and functional differentiation in vitro. Proceedings of the National Academy of Sciences. 81. 3756-3760.

45. Date, K., Matsumoto, K., Shimura, H., Tanaka, M., Nakamura, T. (1997) HGF/NK4 is a specific antagonist for pleiotropic actions of HGF. FEBS Letters. 420. 1-6.

46. Dean, M., Park, M., Vande Woude, G.F. (1987) . Characterization of the rearranged tpr-met oncogene breakpoint. Molecular and Cellular Biology. 7. 921-924.

47. Dedhar, S., Saulnier, R. (1990) Alterations in integrin receptor expression on chemically transformed human cells: specific enhancement of collagen and laminin receptor complexes. Journal of Cell Biology. 110. 481-489.

48. Defrances, M.C., Wolf, H.K., Michalopoulos, G.K., Zarnegar, R. (1992) . The presence of hepatocyte growth factor in the developing rat. Development. 116. 387-395.

49. De Jong, J.S., Van Deist, P.J., Van Der Walk, P., Baak, J.P.A. (1998) Expression of growth factors, growth inhibiting factors, and their receptors in invasive breast cancer. I: An inventory in search of autocrine and paracrine loops. Journal of Pathology. 184. 44-52.

50. De Jong, J.S., Van Deist, P.J., Van Der Walk, P., Baak, J.P.A. (1998) expression of growth factors, growth-inhibiting factors, and their receptors in invasive breast cancer. II: Correlations with proliferation and angiogenesis. Journal of Pathology. 184. 53-57.

51. Devilee, P., Cornelisse, C.J. (1994) Somatic changes in human breast cancer. Biochemical and Biophysical Acta. 1198. 113-130.

52. Devilee, P., Hermans, J., Eyfjord, J., Boorresen, A.L., Lidereau, R., Sobol, H., Borg, A., Cleton-Jansen, A.M., Olah, E., Cohen, B.B., Scherneck, S., Hamann, U., Peterlin, B., Caligo, M., Bignon, Y.J., Maugard, C. (1997) Loss of heterozygosity at 7q31 in breast cancer: results from an International Collaborative Study Group. The Breast Cancer Somatic Genetics Consortium. Genes Chromosomes and Cancer. 18. 193-199.

53. Dickson, R.B. (1996). Biochemical Control of Breast Development. Diseases of the Breast. Harris, J.R., Lippman, M.E., Morrow, M., Hellman, S. 15-25. Philadelphia: Lippincott-Raven.

54. Di Renzo, M.F., Narsimhan, R.P., Olivero, M., Bretti, S., Giordano, S., Medico, E., Gaglia, P., Zara, P., Comoglio, P.M. (1991) Expression of the Met/HGF receptor in normal and neoplastic human tissues. Oncogene. 6. 1997-2003.

55. Di Renzo, M.F., Olivero, M., Ferro, S., Prat, M., Bongarzone, I., Pilotti, S., Belfiore, A., Constantino, A., Vigneri, R., Pieritti, M.A., Comoglio, P.M. (1992) Overexpression of the *c-met*/HGF receptor gene in human thyroid carcinomas. Oncogene. 7. 2549-2553.

56. Di Renzo, M.F., Poulsom, R., Olivero, M., Comoglio, P.M., Lemoine, N.R. (1995) Expression of the Met/hepatocyte growth factor receptor in human pancreatic cancer. Cancer Research. 55. 1129-1138.

57. Dixon, M., Sainsbury, R. Diseases of the breast. 2nd edition. London. Churchill Livingstone, 1998.

58. Dowrick, P., Kenworthy, P., McCann, B., Warn, R. (1993) Circular ruffle formation and closure lead to macropinocytosis in hepatocyte growth factor/scatter factor-treated cells. European Journal of Cell Biology. 61. 44-53.

59. Driouch, K., Briffod, M., Bieche, I., Champeme, M.H., Lidereau, R. (1998) Location of several putative genes possibly involved in human breast cancer progression. Cancer Research. 58. 2081-2086.

60. Dublin, E.A., Barnes, D.M., Wang, D.Y., King, R.J., Levison, D.A. (1993) TGF-α and TGF-β expression in mammary carcinoma. Journal of Pathology. 170. 15-22.

61. Dunsmore, S.E., Rubin, J.S., Kovacs, S.O., Chedid, M., Parks, W.C., Welgus, H.G. (1996) Mechanisms of HGF stimulation of keratinocyte metalloproteinase production. Journal of Biological Chemistry. 271. 24567-24582.

62. Eagles, G., Warn, A., Baff, R.Y., Baillie-Johnston, H., Arakaki, N., Daikuhara, Y., Warn, R.M. (1996) Hepatocyte growth factor/scatter factor is present in most pleural effusion fluids from cancer patients. British Journal of Cancer. 73. 377-381.

63. Ebert, M., Yokoyama, M., Friess, H., Buchler, M.W., Korc, M. (1994) Coexpression of the *c-met* proto-oncogene and hepatocyte growth factor in human pancreatic cancer. Cancer Research. 54. 5775-5778.

64. Ennis, B.W., Valverius, E.M., Lippman, M.E., Bellot, F., Kris, R., Schlessinger, J., Masui, H., Goldenberg., A., Mendelsohn, J., Dickson, RB. (1989) Anti-EGF receptor antibodies inhibit the autocrine stimulated growth of MDA-MB-468 breast cancer cells. Molecular Endocrinology. 3. 1830-1838.

65. Ethier, S.P., Summerfelt, R.M., Cundiff, K.C., Asch, B.B. (1990) The influence of growth factors on the proliferative potential of normal and primary breast cancer-derived human breast epithelial cells. Breast Cancer Research and Treatment. 17. 221-230.

66. Faletto, D.L., Kaplan, D.R., Halverson, D.O., Rosen, E.M., Vande Woude, G.F. (1993) Signal transduction in *c-met* mediated motogenesis. Experimentia Supplement. 65. 107-130.

67. Faletto, D.L., Tsarfaty, I., Kmiecik, T.E., Gonzatti, M., Suzuki, T., Vande Woude, G.F. (1992) Evidence for non-covalent clusters for the *c-met* proto-oncogene product. Oncogene. 7. 1149-1157.

68. Fearon, E.R., Vogelstein, B. (1990) A genetic model for colorectal tumorigenesis. Cell. 61. 759-767.

69. Ferracini, R., Di Renzo, M.F., Scotlandi, K., Baldini, N., Olivero, M., Lollini, P., Cremona, O., Campanacci, M., Comoglio, P.M. (1995) The Met/HGF receptor is overexpressed in human osteosarcomas and is activated by either a paracrine or autocrine circuit. Oncogene. 10. 739-749.

70. Ferracini, R., Longati, P., Naldini, L., Vigna, E., Comoglio, P.M. (1991) Identification of the major autophosphorylation site of the Met/hepatocyte growth factor receptor tyrosine kinase. Journal of Biological Chemistry. 266. 19558-19564.

71. Flavia, Di Renzo, M., Olivero, M., Giacomini, A., Porte, H., Chastre, E., Mirossay, L., Nordlinger, B., Bretti, S., Bottardi, S., Giordano, S., Plebani, M., Gespach, C., Comoglio, P.M. (1995) Overexpression and amplification of the Met/HGF receptor gene during the progression of colorectal cancer. Clinical Cancer Research. 1. 147-154.

72. Folkman, J. (1990) What is the evidence that tumours are angiogenesis dependent? Journal of the National Cancer Institute. 82. 4-6.

73. Folkman, J. (1994) Angiogenesis and breast cancer (editorial). Journal of Clinical Oncology. 12(3). 441-443.

74. Fukuura, T., Miki, C., Inoue, T., Matsumoyo, K., Suzuki, H. (1998) Serum hepatocyte growth factor as an index of disease status of patients with colorectal carcinoma. British Journal of Cancer. 78. 454-459.

75. Furlong, R.A., Takehara, R., Taylor, W.G., Nakamura, T., Rubin, J.S. (1991) Comparison of biological and immunological properties indicates that scatter factor and hepatocyte growth factor are indistinguishable. Journal of Cell Science. 100. 173-177.

76. Fushida, S., Yonemura, Y., Urano, T., Yamaguchi, A., Miyazaki, I., Nakamura, T., Shiku, H. (1993) Expression of hepatocyte growth factor (HGF) and *c-met* gene in human gastric cancer cell lines. International Journal of Oncology. 3. 1067-1070.

77. Gak, E., Taylor, W.G., Chan, A.M., Rubin, J.S. (1992) Processing of hepatocyte growth factor to the heterodimeric form is required for full biological activity. FEBS Letters. 311. 17-21.

78. Gandino, L., Longati, P., Medico, E., Prat, M., Comoglio, P.M. (1994) Phosphorylation of serine 985 negatively regulates the hepatocyte growth factor receptor kinase. Journal of Biological Chemistry. 269. 1815-1820.

79. Gasparini, G., Toi, M., Gion, M., Verderio, P., Dittadi, R., Hanatani, M., Matsubara, I., Vinante, O., Bonoldi, E., Boracchi, P., Gatti, C., Suzuki, H., Tominaga, T. (1997) Prognostic significance of VEGF in node negative breast cancer. Journal of the National Cancer Institute. 89(2). 139-147.

80. Gasparini, G., Weidner, N., Bevilacqua, P., Maluta, S., Dalla-Palma, P., Caffo, O., Barbareschi, M., Boracchi, P., Marubini, E.., Pozza, F. (1994) Tumor microvessel density, p53 expression, tumor size and peritumoral lymphatic vessel invasion are relevant prognostic markers in node negative breast cancer. Journal of Clinical Oncology. 12(3). 454-466.

81. Gaudino, G., Follenzi, A., Naldini, L., Collesi, C., Santoro, M., Gallo, KA., Godowski, PJ., Comoglio, PM. (1994) Ron is a heterodimeric tyrosine kinase receptor activated by the HGF homologue MSP. EMBO Journal. 13. 3524-3532.

82. Gerdes, J., Lemke, H., Baisch, H., Wacjer, H.H., Schwab, U., Stein, H. (1984) Cell cycle analysis of a proliferation-associated human nuclear antigen defined by monoclonal antibody KI-67. Journal of Immunology. 133. 1710-1715.

83. Gherardi, E., Gray, J., Stoker, M., Perryman, M., Furlong, R. (1989) Purification of scatter fector, a fibroblast derived basic protein that modulates epithelial interactions and movement. Proceedings of the National Academy of Sciences. 86. 5844-5848.

84. Gherardi, E., Sharpe, M., Lane, K. (1993) Properties and structure-function relationship of HGF-SF. Experimentia Supplement. 65. 31-48.

85. Gherardi, E., Stoker, M. (1990) Hepatocytes and scatter factor. Nature. 346. 228.

86. Ghoussoub, R.A.D., Dillon, D.A., D'Aquila, T., Rimm, E.B., Fearon, E.R., Rimm, D.L. (1998) Expression of *c-met* is a strong independent prognostic factor in breast carcinoma. Cancer . 82. 1513-1520.

87. Giancotti, F.G., Mainiero, F. (1994) Integrin-mediated adhesion and signalling in tumourigenesis. Biochemical and Biophysical Acta. 1198. 47-64.

88. Giordano, S., Ponzetto, C., Di Renzo, M.F., Cooper, C.S., Comoglio, P.M. (1989) Tyrosine kinase receptor indistinguishable from the *c-met* protein. Nature. 399. 155-156.

89. Giordano, S., Zhen, Z., Medico, E., Gaudino, G., Galimi, F., Comoglio, P.M. (1993) Transfer of motogenic and invasive response to scatter factor/ hepatocyte growth factor by transfection of MET protooncogene. Proceedings of the National Academy of Sciences. 90. 649-653.

90. Gohda, E., Matsunaga, T., Kataoka, H., Takebe, T., Yamamoto, I. (1994) Induction of hepatocyte growth factor in human skin fibroblasts by epidermal growth factor, platelet-derived growth factor and fibroblast growth factor. Cytokine. 6. 633-640.

91. Gohda, E., Tsubouchi, H., Nakayama, H., Hirono, S., Sakiyama, O., Takahashi, K., Miyazaki, H., Hashimoto, S., Daikuhara, Y. (1988) Purification and partial characterisation of hepatocyte growth factor from plasma of a patient with fulminant hepatic failure. Journal of Clinical Investigation. 81. 414-419.

92. Gomyo, Y., Andachi, H., Nagao, K., Ikeguchi, M., Ito, H. (1995) Interphase cytogenetics of gastric carcinoma: fluorescence in situ hybridisation (FISH) applied to cells obtained from formalin-fixed paraffin-embedded tissues. Pathology International. 45. 227-232.

93. Gonzatti-Haces, M., Seth, A., Park, M., Copeland, T., Oroszlan, S., Vande Woude, G.F. (1988) Characterisation of the TPR-MET oncogene p65 and the MET protooncogene p140 protein-tyrosine kinases. Proceedings of the National Academy of Sciences. 85. 21-25.

94. Grant, D.S., Kleinman, H.K., Goldberg, I.D., Bhargava, M.M., Nickoloff, B.J., Kinsella, J.L., Polverini, P., Rosen, E.M. (1993) Scatter factor induces blood vessel formation in vivo. Proceedings of the National Academy of Sciences. 90. 1937-1941.

95. Graziani, A., Gramaglia, D., Dalla Zonca, P., Comoglio, P.M. (1993) Hepatocyte growth factor/scatter factor stimulates the ras-guanine nucleotide exchanger. Journal of Biological Chemistry. 268. 9165-9168.

96. Grey, A.M., Schor, A.M., Rushton, G., Ellis, I., Schor, SL. (1989) Purification of the migration stimulating factor produced by fetal and breast cancer patient fibroblasts. Proceedings of the National Academy of Sciences. 86. 2438-2442.

97. Gullick, W.J., Srinivasan, R. (1998) The type 1 family growth factor receptor family: new ligands and receptors and their role in breast cancer. Breast Cancer Research and Treatment. 52. 43-53.

98. Halaban, R., Rubin, J.S., Funasaka, Y., Cobb, M., Boulton, T., Faletto, D., Rosen, E., Chan, A., Yoko, K., White, W., Cook, C., Moellmann, G. (1992) Met and hepatocyte growth factor/scatter factor signal transduction in normal melanocytes and melanoma cells. Oncogene. 7. 2195-2206.

99. Harris, A.L., Nicholson, S., Sainsbury, R.C., Farndon, J., Wright, C. (1989) EGF receptors in breast cancer: association with early relapse and death, poor response to hormones and interactions with neu. Journal of Steroid Biochemistry. 34. 123-131.

100. Hartmann, G., Naldini, L., Weidner, K.M., Sachs, M., Vigna, E., Comoglio, P.M., Birchmeier, W. (1992) A functional domain in the heavy chain of scatter factor/hepatocyte growth factor binds the *c-met* receptor, induces cell dissociation but not mitogenesis. Proceedings of the National Academy of Sciences. 89. 11574-11578.

101. Hirai, Y., Takebe, K., Takashina, M., Kobayahi, S., Takeichi, M. (1992) Epimorphin: a mesenchymal protein essential for epithelial morphogenesis. Cell. 69. 471-481.

102. Hirota, M., Egami, H., Corra, S., Fujii, H., Chaney, WG., Rizzino, A., Pour, PM. (1993) Production of scatter-factor like activity by a nitrosamine-induced pancreatic cell line. Carcinogenesis. 14. 259-264.

103. Hiscox, S., Jiang, W.G. (1997) Interleukin-12, an emerging anti-tumour cytokine. In Vivo. 11. 125-132.

104. Hiscox, S., Jiang, W.G. (1997) Regulation of endothelial CD44 expression and endothelium-tumour cell interactions by HGF/SF. Biochemical and Biophysical Research Communications. 233. 1-5.

105. Hiscox, S., Jiang, W.G. (1998) HGF/SF regulates the phosphorylation of β-catenin and cell-cell adhesion in cancer cells. Proceedings of the American Association of Cancer Research. 39. 500-501.

106. Hiscox, S., Puntis, M.C.A., Hallett, M.B., Jiang, W.G. (1995) Inhibition of motility and invasion of human colon cancer cells by interleukin-12. Clinical and Experimental Metastasis. 13. 396-404.

107. Horak, E.R., Leek, R., Klenk, N., LeJeune, S., Smith, K., Stuart, N., Greenall, M., Stepniewska, K., Harris, A.L. (1992) Angiogenesis, assessed by platelet/endothelial adhesion molecule antibodies, as indicator of node metastases and survival in breast cancer. Lancet. 340. 1120-1124.

108. Hordijk, P.L., Ten Klooster, J.P., Van Der Kammen, R.A., Michiels, F., Oomen, LCJM, Collard, JG. (1997) Inhibition of invasion of epithelial cells by Tiam1-Rac signalling. Science. 278. 1464-1466.

109. Huang, H., Qian, C., Jenkins, R.B., Smith, D.I. (1998) FISH mapping of YAC clones at human chromosomal band 7q31.2: identification of YACS spanning FRA7G within the common region of LOH in breast and prostate cancer. Genes Chromosomes and Cancer. 21. 152-159.

110. Ignatoski, K.M., Lapointe, A.J., Radany, E.H., Ethier, S.P. (1999) erbB-2 overexpression in human mammary epithelial cells confers growth factor independence. Endocrinology. 140. 3615-3622.

111. Ioachim, E., Kamina, S., Athanassiadou, S., Agnantis, N.J. (1996) The prognostic significance of EGFR, C-erbB-2, Ki-67 and PCNA expression in breast cancer. Anticancer Research. 16. 3141-3147.

112. Itakura, Y., Yamamoto, T., Matsumoto, K., Nakamura, T. (1994) Autocrine stimulation of motility in SBC-5 human lung carcinoma cells by a two-kringle variant of HGF. Cancer Letters. 83. 235-243.

113. Jansen, R.L., Hupperets, P.S., Arends, J.W., Joosten-Achjanie, S.R., Volovics, A., Schouten, H.C., Hillen, H.F. (1998) MIB-1 labelling index is an independent prognostic marker in primary breast cancer. British Journal of Cancer. 78. 460-465.

114. Jeffers, M., Rong, S., Vande Woude, G.F. (1996) Enhanced tumorigenicity and invasion/metastasis by hepatocyte growth factor/scatter factor signalling in human cells concomitant with induction of urokinase proteolysis network. Molecular and Cellular Biology. 16. 1115-1125.

115. Jeffers, M., Rong, S., Vande Woude, G.F. (1996) Hepatocyte growth factor/scatter factor-Met signalling in tumorigenicity and invasion/metastasis. Journal of Molecular Medicine. 74. 505-513.

116. Jenkins, R.B., Qian, J., Lee, H.K., Huang, H., Hirasawa, K., Bostwick, D.G., Proffitt, J., Wilber, K., Lieber, M.M., Liu, W., Smith, D.I. (1998) A molecular cytogenetic analysis of 7q31 in prostate cancer. Cancer Research. 58. 759-766.

117. Jiang, W.G., Bryce, R.P., Horrobin, D.F. (1998) Essential fatty acids, the molecular mechanisms of their anticancer actions and clinical implications. Critical Reviews in Oncology and Hematology. 27. 179-209.

118. Jiang, W.G., Hallett, M.B., Puntis, M.C.A. (1993) Hepatocyte growth factor/scatter factor, liver regeneration and cancer metastasis. British Journal of Surgery. 80. 1368-1373.

119. Jiang, W.G., Hiscox, S., Hallett, M.B., Horrobin, DF., Mansel, RE., Puntis, MCA. (1995) Regulation of expression of E-cadherin on human cancer cells by gamma linolenic acid. Cancer Research. 55. 5043-5048.

120. Jiang, W.G., Hiscox, S., Hallett, M.B., Bryce, R., Horrobin, D.F., Mansel, R.E., Puntis, M.C.A. (1996) Inhibition of membrane ruffling and ezrin translocation by gamma linolenic acid. International Journal of Oncology. 9. 279-284.

121. Jiang, W.G., Hiscox, S., Hallett, M.B., Scott, C., Horrobin, D.F., Puntis, M.C.A. (1995) Inhibition of HGF-induced motility and in vitro invasion of human colon cancer cells by gamma-linolenic acid. British Journal of Cancer. 71. 744-752.

122. Jiang, W., Hiscox, S., Matsumoto, K., Nakamura, T. (1999) Hepatocyte growth factor/scatter factor, its molecular, cellular and clinical implications in cancer. Critical Reviews in Oncology and Hematology. 29. 209-248.

123. Jiang, W.G., Hiscox, S., Nakamura, T., Hallett, MB., Puntis, MCA., Mansel, RE. (1996) HGF/SF induces tyrosine phosphorylation of focal adhesion kinase (FAK) and paxilin and enhances cell-matrix interactions. Oncology Reports. 3. 819-823.

124. Jiang, W.G., Hiscox, S., Singhrao, S.K., Nakamura, T., Puntis, MCA., Hallett, MB. (1995) Inhibition of HGF/SF induced membrane ruffling and cell motility by transient elevation of cytosolic free calcium. Experimental Cell Research. 200. 424-433.

125. Jiang, W.G., Lloyds, D., Puntis, M.C., Nakamura, T., Hallett, M.B. (1993) Regulation of spreading and growth of colon cancer cells by hepatocyte growth factor. Clinical and Experimental Metastasis. 11. 235-242.

126. Jin, L., Fuchs, A., Schnitt, S.J., Yao, Y., Joseph, A., Lamszus, K., Park, M., Goldberg, I.D., Rosen, E.M. (1997) Expression of scatter factor and *c-met* receptor in benign and malignant breast tissue. Cancer . 79. 749-760.

127. Johnson, M., Koukoulis, G., Matsumoto, K., Nakamura, T., Iyer, A. (1993) Hepatocyte growth factor induces proliferation and morphogenesis in non-parenchymal liver cells. Hepatology. 17. 1052-1061.

128. Joplin, R., Hishida, T., Tsubouchi, H., Daikuhara, Y., Ayres, R., Neuberger, J.M., Strain, A.J. (1992) Human intrahepatic biliary cells proliferate in vitro in reponse to human HGF. Journal of Clinical Investigation. 90. 1284-1289.

129. Joseph, A., Weiss, G.H., Jin, L., Fuchs, A., Chowdhury, S., O'Shaugnessy, P., Goldberg, I.D., Rosen, E.M. (1995) Expression of scatter factor in human bladder carcinoma. Journal of the National Cancer Institute. 87. 372-377.

130. Kalamati, T., Thirunavukarasu, B., Wallace, A., Holder, N., Brooks, R.F., Nakamura, T., Stoker, M., Gherardi, E., Buluwela, A. (1992) Downregulation of scatter factor in MRC5 fibroblasts by epithelial derived cells: a model for scatter factor modulation. Journal of Cell Science. 101. 323-332.

131. Kato, S., Ishii, T., Hara, H., Sugiura, N., Kimata, K., Akamatsu, N. (1994) HGF immobilised onto culture substrates through heparin and matrigel enhances DNA synthesis in primary rat hepatocytes. Experimental Cell Research. 211. 53-58.

132. Kawaguchi, T., Qin, L., Shimomura, T., Kondo, J., Matsumoto, K., Denda, K., Kitamura, N. (1997) Purification and cloning of HGF activator inhibitor type 2, a Kunitz-type serine protease inhibitor. Journal of Biological Chemistry. 272. 27558-27564.

133. Kawakami-Kimura, N., Narita, T., Ohmori, K.,. Yoneda, T., Matsumoto, K., Nakamura, T., Kannagi, R. (1997) Involvement of HGF in increased integrin expression on HepG2 cells triggered by adhesion to endothelial cells. British Journal of Cancer. 75. 47-53.

134. Kenworthy, P., Dorwick, P., Baillie-Johnson, H., McCann, B., Tsubouchi, H., Arakaki, N., Daikuhara, Y., Warn, R.M. (1992) The presence of scatter factor in patients with metastatic spread to the pleura. British Journal of Cancer. 66. 243-247.

135. Kerangueven, F., Noguchi, T., Coulier, F., Allione, F., Wargniez, V., Simony-Lafontaine, J., Longy, M., Jacqemier, J., Sobol, H., Eisinger, F., Birnbaum, D. (1997) Genome-wide search for loss of heterozygosity shows extensive diversity of human breast carcinomas. Cancer Research. 57. 5469-5474.

136. Kim, K.J., Li, B., Winer, J., Armanini, M., Gillett, N., Phillips, H.S., Ferrara, N. (1993) Inhibition of vascular endothelial growth-factor induced angiogenesis suppresses tumour growth in vivo. Nature. 362. 841-844.

137. Kioke, M., Takeuchi, S., Yokota, J., Park, S., Hatta, Y., Miller, C.W., Tsuruoka, N., Koeffler, H.P. (1997) Frequent loss of heterozygosity in the region of the D7S523 locus in advanced ovarian cancer. Genes Chromosomes and Cancer. 19. 1-5.

138. Kitamura, N. (1993) Scatter factor and cell motility. Gan-To-Kagaku-Ryoho. 20. 410-416.

139. Klagsburn, M., D'Amore, P.A. (1991) Regulators of angiogenesis. Annual Reviews of Physiology. 53. 217-239.

140. Knudson, A.G.J. (1985) Hereditary cancer, oncogenes, and anti-oncogenes. Cancer Research. 45. 1437-1443.

141. Komada, M., Kitamura, N. (1993) The cell dissociation and motility triggered by scatter factor/hepatocyte growth factor are mediated through the cytoplasmic domain of the *c-met* receptor. Oncogene. 8. 2381-2390.

142. Konishi, T., Takehara, T., Tsuji, T., Ohsato, K., Matsumoto, K., Nakamura, T. (1991) Scatter factor from human embryonic lung fibroblast is probably identical to hepatocyte growth factor. Biochemical and Biophysical Research Communications. 180. 765-773.

143. Koochekpour, S., Jeffers, M., Rulong, S., Taylor, G., Klineberg, E., Hudson, EA., Resau, JH., Vande Woude, GF. (1997) Met and HGF/SF expression in human gliomas. Cancer Research. 57. 5391-5398.

144. Kuniyasu, H., Yasui, W., Kitadai, Y., Yokozaki, H., Ito, H., Tahara, E. (1992) Frequent amplification of the *c-met* gene in schirrous type gastric cancer. Biochemical and Biophysical Research Communications. 189. 227-232.

145. Kuniyasu, H., Yasui, W., Yokozaki, H., Akagi, M., Akama, Y., Kitahara, K., Fujii, K., Tahara, E. (1994) Frequent loss of heterozygosity of the long arm of chromosome 7 is closely associated with progression of human gastric carcinomas. International Journal of Cancer. 59. 597-600.

146. Kuniyasu, H., Yasui, W., Yokozaki, H., Kitadai, Y., Tahara, E. (1993) Aberrant expression of *c-met* mRNA in human gastric carcinomas. International Journal of Cancer. 55. 72-75.

147. Lamszus, K., Jin, L., Fuchs, A., Shi, E., Chowdhury, S., Yao, Y., Polverini, P.J., Laterra, J., Goldberg, I.D., Rosen, E.M. (1997) Scatter factor stimulates tumour growth and tumour angiogenesis in human breast cancers in the mammary fat pads of nude mice. Laboratory Investigation. 76. 339-353.

148. Lamszus, K., Joseph, A., Jin, L., Yao, Y., Chowdhury, S., Fuchs, A., Polverini, PJ., Goldberg, ID., Rosen, EM. (1996) Scatter factor binds to thrombospondin and other extracellular matrix components. American Journal of Pathology. 149. 805-819.

149. Laterra, J., Nam, M., Rosen, E., Rao, JS., Lamszus, K., Goldberg, ID., Johnston, P. (1997) Scatter factor/hepatocyte growth factor gene transfer enhances glioma growth and angiogenesis in vivo. Laboratory Investigation. 76. 565-577.

150. Laterra, J., Rosen, E.M., Nam, M., Rangathan, S., Fielding, K., Johnston, P. (1997) SF/HGF expression enhances human glioblastoma tumorigenicity and growth. Biochemical and Biophysical Research Communications. 255. 743-747.

151. Latil, A., Cussenot, O., Fournier, G., Baron, J.C., Lidereau, R. (1995) Loss of heterozygosity at 7q31 is a frequent and early event in prostate cancer. Clinical Cancer Research. 1. 1385-1389.

152. Liang, T.J., Reid, A.E., Xavier, R., Cardiff, R.D., Wang, T.C. (1996) Transgenic expression of the tpr-met oncogene leads to the development of mammary hyperplasia and tumours. Journal of Clinical Investigation. 97. 2872-2877.

153. Lin, J.C., Scherer, S.W., Tougas, L., Traverso, G., Tsui, L.C., Andrulis, I.L., Jothy, S., Park, M. (1996) Detailed deletion mapping with refined physical map of 7q31 localises a putative tumour suppressor gene for breast cancer in the region of MET. Oncogene. 13. 2001-2008.

154. Ling-Chun, C., Matsumara, K., Deng, G., Kirisu, W., Ljung, B.M., Lerman, M.I., Waldman, F.M., Smith, H.S. (1994) Deletion of two separate regions on chromosome 3p in breast cancers. Cancer Research. 54. 3021-3024.

155. Liu, C., Park, M., Tsao, M.S. (1992) Overexpression of *c-met* proto-oncogene but not epidermal growth factor receptor or c-erbB-2 in primary human colorectal carcinomas. Oncogene. 7. 181-185.

156. Liu, S.I., Lui, W.Y., Mok, K.T., Wu, CW., Chi, CW. (1997) Effect of HGF on cell cycle and *c-met* expression in human gastric cancer cells. Anticancer Research. 17. 3575-3580.

157. Liu, Y., Lin, L., Zarnegar, R. (1994) Modulation of hepatocyte growth factor gene expression by estrogen in mouse ovary. Molecular and Cellular Endocrinology. 104. 173-181.

158. Lokker, N.A., Mark, M.R., Luis, E.A., Bennett, G.L., Robbins, K.A., Baker, J.B., Godowsski, P.J. (1992) Structure-function analysis of hepatocyte growth factor: identification of variants that lack mitogenic activity yet retain high affinity receptor binding. EMBO Journal. 11. 2503-2510.

159. Longati, P., Bardelli, A., Ponzetto, C., Naldini, L., Comoglio, P.M. (1994) Tyrosines1234-1235 are critical for activation of the tyrosine kinase encoded by the MET proto-oncogene (HGF receptor). Oncogene. 9. 49-57.

160. Lupu, R., Cardillo, M., Cho, C., Harris, L., Hijazi, M., Perez, C., Rosenberg, K., Yang, D., Tang, C. (1996) The significance of heregulin in breast cancer tumour progression and drug resistance. Breast Cancer Research and Treatment. 38. 57-66.

161. Lyon, M., Deakin, J.A., Mizuno, K., Nakamura, T., Gallagher, J.T. (1994) Interaction of hepatocyte growth factor with heparan sulfate. Elucidation of the major heparan sulfate structural determinants. Journal of Biological Chemistry. 269. 11216-11223.

162. MacDonald, N.J., Steeg, P.S. (1993) Molecular basis of tumour metastasis. Cancer Surveys. 16. 175-199.

163. Marson, L.P., Miller, W.R., Dixon, J.M. (1998) Angiogenesis and breast cancer. The Breast. 7. 299-307.

164. Martin, L., Green, B., Renshaw, C., Lowe, D., Rudland, P., Leinster, S.J., Winstanley, J. (1997) Examining the technique of angiogenesis assessment in invasive breast cancer. British Journal of Cancer. 76(8). 1046-1054.

165. Matsumoto, K., Date, K., Ohmichi, H., Nakamura, T. (1996) HGF in lung morphogenesis and tumor invasion: role as a mediator in epithelium-mesenchyme and tumor-stroma interactions. Cancer Chemotherapy Pharmacology. 38. S42-47.

166. Matsumoto, K., Date, K., Shimura, H., Nakamura, T. (1996) Acquisition of invasive phenotype in gallbladder cancer-cells via mutual interaction of stromal fibroblasts and cancer cells as mediated by HGF. Japanese Journal of Cancer Research. 87. 702-710.

167. Matsumoto, K., Hashimoto, K., Yoshikawa, K., Nakamura, T. (1991) Marked stimulation of growth and motility of human keratinocytes by HGF. Experimental Cell Research. 196. 114-120.

168. Matsumoto, K., Horikoshi, M., Rikimaru, K., Enomoto, S. (1989) A study of an in vitro model for invasion of oral squamous cell carcinoma. Journal of Oral Pathology and Medicine. 18. 498-501.

169. Matsumoto, K., Matsumoto, K., Nakamura, T., Kramer, R.H. (1994) Hepatocyte growth factor/scatter factor induces tyrosine phosphorylation of focal adhesion kinase (p125FAK) and promotes migration and invasion by oral squamous carcinoma cells. Journal of Biological Chemistry. 269 . 31807-31813.

170. Matsumoto, K., Okazaki, H., Nakamura, T. (1992) Upregulation of hepatocyte growth factor gene expression by interleukin-1 in human skin fibroblasts. Biochemical and Biophysical Research Communications. 188. 235-243.

171. Matsumoto, K., Okazaki, H., Nakamura, T. (1995) Novel function of prostaglandins as inducers of gene expression of HGF and putative mediators of tissue regeneration. Journal of Biochemistry. 117. 458-464.

172. Matsumoto, K., Tajima, H., Hamanoue, M., Kohno, S., Kinoshita, T., Nakamura, T. (1992) Identification and characterisation of injurin, an inducer of the gene expression of HGF. Proceedings of the National Academy of Sciences. 89. 3800-3804.

173. Matsumoto, K., Tajima, H., Okazaki, H., Nakamura, T. (1993) Heparin as an inducer of hepatocyte growth factor. Journal of Biochemistry. 114. 820-826.

174. Matsumoto, K., Takehara, T., Inoue, H., Hagiya, M., Shimizu, S., Nakamura, T. (1991) Deletion of kringle domains or the N-terminal hairpin structure in HGF results in marked decreases in related biological activities. Biochemical and Biophysical Research Communications. 181. 691-699.

175. McCune, B.K., Mullin, B.R., Flanders, C.K., Jaffurs, WJ., Mullen, LT., Sporn, MB. (1991) Localisation of TGF-beta isotypes in lesions of the human breast. Human Pathology. 23. 13-20.

176. Meiners, S., Brinkmann, V., Naundorf, H., Birchmeier, W. (1998) Role of morphogenic factors in metastasis of mammary carcinoma cells. Oncogene. 16. 9-20.

177. Michalopoulos, G., Houck, K.A., Dolan, M.L., Luetteke, N.C. (1984) Control of hepatocyte replication by two serum factors. Cancer Research. 44. 4414-4419.

178. Miyazaki, M., Bai, L., Taga, H., Hirai, H., Sato, J., Namba, M. (1991) Expression of liver-specific functions and secretion of a hepatocyte growth factor by a newly established rat hepatoma cell line growing in a chemically-defined serum free medium. Research in Experimental Medicine. 191. 297-307.

179. Miyazawa, K., Kitamura, A., Naka, D., Kitamura, N. (1991) An alternatively processed mRNA generated from human hepatocyte growth factor gene. European Journal of Biochemistry. 197. 15-22.

180. Miyazawa, K., Shimomura, T., Kitamura, N. (1996) Activation of HGF in the injured tissues is mediated by hepatocyte growth-factor activator. Journal of Biological Chemistry. 271. 3615-3618.

181. Miyazawa, K., Shimomura, T., Naka, D., Kitamura, N. (1994) Proteolytic activation of hepatocyte growth factor in response to tissue injury. Journal of Biological Chemistry. 269. 8966-8970.

182. Miyazawa, K., Trubouchi, H., Naka, D., Takahashi, K., Okigaki, M., Arakaki, N., Nakayama, H., Hirono, S., Sakaiyama, O., Takahashi, K., Gohda, E., Daikuhara, Y., Kitamura, N. (1989) Molecular cloning and sequence analysis of cDNA for human hepatocyte growth factor. Biochemical and Biophysical Research Communications. 163. 967-973.

183. Mizuno, K., Higuchi, O., Tajima, H., Yonemasu, T., Nakamura, T. (1993) Cell density-dependent regulation of hepatocyte growth factor receptor on adult rat hepatocytes in primary culture. Journal of Biochemistry. 114. 96-102.

184. Mizuno, K., Nakamura, T. (1993). Molecular characteristics of HGF and the gene, and its biochemical aspects. Experimentia Supplement. 65. 1-29.

185. Mizuno, K., Takehara, T., Nakamura, T. (1992) . Proteolytic activation of a single-chain precursor of hepatocyte growth factor by extracellular serine protease. Biochemical and Biophysical Research Communications. 189. 1631-1638.

186. Moghul, A., Lin, L., Beedle, A., Kanbour-Shakir, A., DeFrances, M.C., Liu, Y., Zarnegar, R. (1994) Modulation of *c-met* proto-oncogene (HGF receptor) mRNA abundance by cytokines and hormones: evidence for rapid decay of the 8kb *c-met* transcript. Oncogene. 9. 2045-2052.

187. Montesano, R., Matsumoto, K., Nakamura, T., Orci, L. (1991) Identification of a fibroblast-derived epithelial morphogen as HGF. Cell. 67. 901-908.

188. Montesano, R., Schaller, G., Orci, L. (1991) Induction of epithelial tubular morphogenesis in vitro by fibroblast-derived soluble factors. Cell. 66. 697-711.

189. Moore, M.W., Klein, R.D., Farinas, I., Sauer, H., Armanini, M., Phillips, H., Reichardt, L.F., Ryan, A.M., Carver-Moore, K., Rosenthal, A. (1996) Renal and neuronal abnormalities in mice lacking GDNF. Nature. 382. .

190. Morimoto, A., Tada, K., Nakayama, Y., Kohno, K., Naito, S., Ono, M., Kuwano, M. (1994) Cooperative roles of hepatocyte growth factor and plasminogen activator in tubular morphogenesis by human microvascular endothelial cells. Japanese Journal of Cancer Research. 85. 53-62.

191. Nagai, H., Negrini, M., Carter, S.L., Gillum, D.R., Rosenberg, A.L., Schwartz, G.F., Croce, C.M. (1995) Detection and cloning of a common region of loss of heterozygosity at chromosome 1p in breast cancer. Cancer Research. 55. 1752-1757.

192. Naidu, Y.M., Rosen, E.M., Zitnik, R., Goldberg, I., Park, M., Naujokas, M., Polverini, PJ., Nickoloff, BJ. (1994) Role of scatter factor in the pathogenesis of AIDS-related Kaposi sarcoma. Proceedings of the National Academy of Sciences. 91. 5281-5285.

193. Naka, D., Ishi, T., Yoshiyama, Y., Miyazawa, K., Hara, H., Hishida, T., Kitumura, N. (1992) Activation of hepatocyte growth factor by proteolytic conversion of a single chain form to a heterodimer. Journal of Biological Chemistry. 267. 20114-20119.

194. Nakamura, T. (1991) Structure and function of hepatocyte growth factor. Progress in Growth Factor Research. 3. 67-85.

195. Nakamura, T., Matsumoto, K., Kiritoshi, A., Tune, Y., Nakamura, T. (1997) Induction of HGF by fibroblasts by tumor-derived factors effects invasive growth of tumour cells: in vitro analysis of tumor-stromal interactions. Cancer Research. 57. 3305-3313.

196. Nakamura, T., Nawa, K., Ichihara, A. (1984) Partial purification of characterisation of hepatocyte growth factor from serum of hepatectomised rats. Biochemical and Biophysical Research Communications. 122. 1450-1459.

197. Nakamura, T., Nawa, K., Ishihara, A., Kaise, N., Nishino, T. (1987) Purification and subunit structure of hepatocyte growth factor from rat platelets. FEBS Letters. 224. 311-316.

198. Nakamura, T., Nishizawa, T., Hagiya, M., Seki, T., Shimonishi, M., Sugiura, A., Tashiro, K., Shimizu, S. (1989) Molecular cloning and expression of human hepatocyte growth factor. Nature. 342. 440-443.

199. Nakamura, Y., Morishita, R., Higaki, J., Kida, I., Aoki, M., Moriguchi, A., Yamada, K., Hayashi, S., Yo, Y., Nakano, H., Matsumoto, K., Nakamura, T., Ogihara, T. (1996) HGF is a novel member of the endothelium-specific growth factors: additive stimulatory effect of HGF with basic FGF but not with VEGF. Journal of Hypertension. 14. 1067-1072.

200. Nakamura, Y., Morishita, R., Higaki, J., Kida, I., Aoki, M., Moriguchi, A., Yamada, K., Hayashi, S., Yo, Y., Matsumoto, K., Nakamura, T., Ogihara, T. (1995) Expression of local hepatocyte growth factor system in vascular tissues. Biochemical and Biophysical Research Communications. 215. 483-488.

201. Naldini, L., Tamagnone, L., Vigna, E., Sachs, M., Hartmann, G., Birchmeier, W., Daikuhara, Y., Tsubouchi, H., Blasi, F., Comoglio, P.M. (1992) Extracellular proteolytic cleavage by urokinase is required for activation of hepatocyte growth factor/scatter factor. EMBO Journal. 11. 4825-4833.

202. Naldini, L., Vigna, A., Narsimhan, R.P., Gandino, G., Zarnegar, R., Michalopoulos, G.K., Comoglio, P.M. (1991) Hepatocyte growth factor (HGF) stimulates the tyrosine kinase activity of the receptor encoded by the proto-oncogene *c-met*. Oncogene. 6. 501-504.

203. Naldini, L., Vigna, E., Bardelli, A., Follenzi, A., Galimi, F., Comoglio, P.M. (1995) Biological activation of pro-HGF by urokinase is controlled by a stoichiometric reaction. Journal of Biological Chemistry. 270. 603-611.

204. Naldini, L., Vigna, E., Ferracini, R., Longati, P., Gandino, L., Prat, M., Comoglio, P.M. (1991) The tyrosine kinase encoded by the MET proto-oncogene is activated by autophosphorylation. Molecular and Cellular Biology. 11. 1793-1803.

205. Naldini, L., Weidner, K.M., Vigna, E., Gaudino, G., Bardelli, A., Ponzetto, C., Narsimhan, R., Hartmann, G., Zarnegar, R., Michalopoulos, G., Birchmeier, W., Comoglio, P.M. (1991) Scatter factor and hepatocyte growth factor are indistinguishable ligands for the MET receptor. EMBO Journal. 10. 2867-2878.

206. Narita, T., Mitsuoka, C., Kimura, N., Toi, M., Nakashio, T., Sekiguchi, K., Iwanari, H., Kannagi, R. (1997) Increased concentration of HGF in the sera of patients with gastrointestinal cancer. Oncology Reports. 4/6. 1239-1241.

207. Nicholson, R.I., McClelland, R.A., Gee, J.M.W., Manning, D.L., Cannon, P., Robertson, J.F.R., Ellis, I.O., Blamey, R.W. (1994) EGF receptor expression in breast cancer: associations with response to endocrine therapy. Breast Cancer Research and Treatment. 29. 117-125.

208. Niranjan, B., Buluwela, L., Yant, J., Perusinghe, N., Atherton, A., Phippard, D., Dale, T., Gusterson, B., Kalamati, T. (1995) HGF/SF: a potent cytokine for mammary growth, morphogenesis and development. Development. 121. 2897-2908.

209. Nishizuka, S., Tamura, G., Terashima, M., Satodate, R. (1997) Commonly deleted region on the long arm of chromosome 7 in differentiated adenocarcinoma of the stomach. British Journal of Cancer. 76. 1567-1571.

210. Noji, S, Tashiro, K., Koyama, E., Nohno, T., Ohyama, K., Taniguchi, S., Nakamura, T. (1990) Expression of hepatocyte growth factor gene in endothelial and Kupfer cells of damaged rat livers, as revealed by in situ hybridisation. Biochemical and Biophysical Research Communications. 173. 42-47.

211. Oehler, M.K., Rehn, M., Kristen, P., Sutterlin, M., Caffier, H. (1997) Correlation of the EGF-receptor with cell kinetic and classical prognostic factors in breast cancer. Anticancer Research. 17. 3137-3140.

212. Ogata, T., Ayusawa, D., Namba, M., Takahashi, E., Oshimura, M., Oishi, M. (1993) Chromosome 7 suppresses indefinite division of nontumorigenic immortalised human fibroblast cell lines KMST-6 and SUSM-1. Molecular and Cellular Biology. 13. 6036-6043.

213. Ohmichi, H., Koshimizu, U., Matsumoto, K., Nakamura, T. (1998) HGF acts as a mesenchymal derived morphogenic factor during fetal lung development. Development. 125. 1315-1324.

214. Okano, Y., Mizuno, K., Osada, S., Nakamura, T., Nozawa, Y. (1993) Tyrosine phosphorylation of phospholipase-γ in *c-met*/HGF receptor stimulated hepatocytes: comparison with HepG2 hepatocarcinoma cells. Biochemical and Biophysical Research Communications. 190. 842-848.

215. Park, M., Dean, M., Cooper, C.S., Schmidt, M., O'Brien, S.J., Blair, D.G., Vande Woude, G.F. (1986) Mechanism of met oncogene activation. Cell. 45. 895-904.

216. Park, M., Dean, M., Kaul, K., Braun, M.J., Gonda, M.A., Vande Woude, G.F. (1987) Sequence of met protooncogene cDNA has features characteristic of the tyrosine kinase family of growth factor receptors. Proceedings of the National Academy of Sciences. 84. 6379-6383.

217. Pasdar, M., Li, Z., Marreli, M. (1997) Inhibition of junction assembly in cultured epithelial cells by HGF/SF is concomitant with increased stability and altered phosphorylation of the soluble junctional molecules. Cell Growth and Differentiation. 8. 451-462.

218. Pelicci, G., Giordano, S., Zhen, Z., Salcini, A.E., Lanfrancone, L., Bardelli, A., Panayotou, G., Waterfield, M.D., Ponzetto, C., Pelicci, P.G. (1995) The motogenic and mitogenic responses to HGF are amplified by the Shc adaptor protein. Oncogene. 10. 1631-1638.

219. Pepper, M.S., Matsumoto, K., Nakamura, T., Orci, L., Montesano, R. (1992) HGF increases urokinase-type plasminogen activator (u-PA) and u-PA receptor in MDCK epithelial cells. Journal of Biological Chemistry. 267. 20493-20496.

220. Peters, K., Werner, S, Liao, X., Wert, S., Whitsett, J., Williams, L. (1994) Targeted expression of a dominant negative FGF receptor blocks branching morphogenesis and epithelial differentiation in mouse lung. EMBO Journal. 13. 3296-3301.

221. Picard, O., Rolland, Y., Poupon, M.F. (1986) Fibroblast-dependant tumorigenicity of cells in nude mice: implications for implantation of metastases. Cancer Research. 46. 3290-3294.

222. Pichel, J.G., Shen, L., Sheng, H.Z., Granholm, A.C., Drago, J., Grinberg, A., Lee, E.J., Huang, S.P., Saarma, M., Hoffer, B.J., Sariola, H., Westphal, H. (1996) Defects in enteric innervation and kidney development in mice lacking GDNF. Nature. 382. 73-76.

223. Pisters, L.L., Troncoso, P., Zhau, H.E., Li, W., Von Eschenbach, A.C., Chung, L.W. (1995) *C-met* proto-oncogene expression in benign and malignant human prostate. Journal of Urology. 154. 293-298.

224. Plaschke-Schlutter, A., Behrens, J., Gherardi, E., Birchmeier, W. (1995) Characterisation of the SF/HGF gene promoter. Journal of Biological Chemistry. 270. 830-836.

225. Ponzetto, C., Bardelli, A., Maina, F., Longati, P., Panayotou, G., Dhand, R., Waterfield, M.D., Comoglio, P.M. (1993) A novel recognition motif for phosphatidylinositol 3-kinase binding mediates its association with the HGF/SF receptor. Molecular and Cellular Biology. 13. 4600-4608.

226. Ponzetto, C., Bardelli, A., Zhen, Z., Maina, F., Dalla Zonca, P., Giordano, S., Graziani, A., Panayotou, G., Comoglio, P.M. (1994) A multifunctional docking site mediates signalling and transformation by the hepatocyte growth factor/scatter factor receptor family. Cell. 77. 261-271.

227. Porschen, R., Classen, S., Piontek, M., Borchard, F. (1994) Vascularisation of carcinomas of the oesophagus and its correlation with tumour proliferation. Cancer Research. 54. 587-591.

228. Porschen, R., Langen, C., Kriegel, A., Lohe, B., Borchard, F. (1989) Critical evaluation of histochemical and immunohistochemical methods for the demonstration of vascular supply in rectal and oesophageal cancer. British Journal of Cancer. 60. 299-302.

229. Prat, M., Narsimhan, R.P., Crepaldi, T., Nicotra, M.R., Natali, P.G., Comoglio, P.M. (1991) The receptor encoded by the human *c-met* oncogene is expressed in hepatocytes, epithelial cells and solid tumours. International Journal of Cancer. 49. 323-328.

230. Rahimi, N., Saulnier, R., Nakamura, T., Park, M., Elliott, B. (1994) Role of hepatocyte growth factor in breast cancer: A novel mitogenic factor secreted by adipocytes. DNA &Cell Biology. 13. 1189-1197.

231. Railo, M., Nordling, S., von Boguslawsky, K., Leivonen, M., Kyllonen, L., von Smitten, K. (1993) Prognostic value of Ki-67 immunolabelling in primary operable breast cancer. British Journal of Cancer. 68. 579-583.

232. Ram, T.G., Kokeny, K.E., Dilts, C.A., Ethier, S.P. (1995) Mitogenic activity of neu differentiation factor/heregulin mimics that of EGF and IGF-1 in human mammary epithelial cells. Journal of Cellular Physiology. 163. 589-596.

233. Ramadori, G., Neubauer, K., Odenthal, M., Nakamura, T., Knittel, T., Schwogler, S., Meyer zum Buschenfelde, K.H. (1992) The gene of HGF is expressed in fat storing cells of rat liver and is downregulated during cell growth and by TGF-beta. Biochemical and Biophysical Research Communications. 183. 739-742.

234. Rao, U.N.M., Sonmez Alpan, E., Michalopoulos, G.K. (1997) Hepatocyte growth factor and *c-met* in benign and malignant nerve sheath tumours. Human Pathology. 28. 1066-1070.

235. Reichmann, E., Ball, R., Groner, B., Friis, R.R. (1989) New mammary epithelial and fibroblast cell clones in coculture form structures competent to differentiate functionally. Journal of Cell Biology. 108. 1127-1138.

236. Reiss, M., Barcellos-Hoff, M.H. (1997) Transforming growth factor-beta in breast cancer: a working hypothesis. Breast Cancer Research and Treatment. 45. 81-95.

237. Robinson, S.D., Silberstein, G.B., Roberts, A.B., Flanders, K.C., Daniel C.W. (1991) Regulated expression and growth inhibitory effects of TGF-beta isoforms in mouse mammary gland development. Development. 113. 867.

238. Roletto, F., Galvani, A.P., Cristiani, C., Valsasina, B., .Landonio, A., Bertolero, F. (1996) Basic fibroblast growth factor stimulates HGF/SF secretion by human mesenchymal cells. Journal of Cell Physiology. 166. 105-111.

239. Rong, S, Bodescot, M., Blair, D., Nakamura, T., Mizuno, K., Park, M., Chan, A., Aaronson, S., Vande Woude, G.F. (1992) Tumorigenicity of the met proto-oncogene and gene for hepatocyte growth factor. Molecular and Cellular Biology. 12. 5152-5158.

240. Rong, S., Jeffers, M., Resau, J.H., Tsarfaty, I., Oskarsson, M., Vande Woude, G.F. (1993) Met expression and sarcoma tumorigenicity. Cancer Research. 53. 5355-5360.

241. Ronsin, C., Muscatelli, F., Mattei, M.G. (1993) A novel putative receptor tyrosine kinase of the met family. Oncogene. 8. 1195-1202.

242. Rosen, E.M., Goldberg, I.D. (1995) Scatter factor and angiogenesis. Advances in Cancer Research. 67. 257-279.

243. Rosen, E.M., Goldberg, I.D., Jaken, S., Grant, D.S. (1990) Regulation of endothelial cell migration and capillary like tube formation by scatter factor. Circulation. 82. 699.

244. Rosen, E.M., Goldberg, I.D., Liu, D., Setter, E., Donovan, M.A., Bhargava, M., Reiss, M., Kacinski, B.M. (1991) Tumour necrosis factor stimulates epithelial cell motility. Cancer Research. 57. 5315-5321.

245. Rosen, E.M., Grant, D., Kleinman, H., Jaken, S., Donovan, M.A., Setter, E., Luckett, P.M., Carley, W., Bhargava, M., Goldberg, I.D. (1991) Scatter factor stimulates migration of vascular endothelium and capillary-like tube formation. Experimentia Supplement. 59. 76-88.

246. Rosen, E.M., Joseph, A., Jin, L., Rockwell, S., Elias, J.A., Knesel, J., Wines, J., McClellan, J., Kluger, M.J., Goldberg, I.D., Zitnik, R. (1994) Regulation of scatter factor production via a soluble inducing factor. Journal of Cell Biology. 127. 225-234.

247. Rosen, E.M., Knesel, J., Goldberg, I.D., Bhargava, M., Joseph, A., Zitnik, R., Wines, J., Kelley, M., Rockwell, S. (1994) Scatter factor modulates the metastatic phenotype of the EMT6 mouse mammary tumour. International Journal of Cancer. 57. 706-714.

248. Rosen, E.M., Lamszus, K., Laterra, J., Polverini, P.J., Rubin, J.S., Goldberg, I.D. (1997). HGF/SF in angiogenesis. Ciba Foundation Symposium. 212. 215-229.

249. Rosen, E.M., Meromsky, L., Goldberg, I., Bhargava, M., Setter, E., Vinter, D.W., Goldberg, I.D. (1990) Purified scatter factor stimulates epithelial and vascular endothelial cell migration. Journal of Cell Science. 96. 639-649.

250. Rosen, E.M., Meromsky, L., Setter, E., Vinter, D.W., Goldberg, I.D. (1990) Smooth-muscle derived factor stimulates motility of human tumour cells. Invasion Metastasis. 10. 49-64.

251. Rosen, E.M., Zitnik, R.J., Elias, J.A., Bhargava, M.M., Wines, J., Goldberg, I.D. (1993) The interaction of HGF-SF with other cytokines in tumor invasion and metastasis. Experimentia Supplement. 65. 301-310.

252. Rubin, J.S., Chan, A.M.L., Bottaro, D.P., Burgess, W.S., Taylor, W.G., Cech, A.C., Hirschfield, D.W., Wong, J., Miki, T., Finch, P.W., Aaronson, S.A. (1991) A broad spectrum human lung fibroblast derived mitogen is a variant of hepatocyte growth factor. Proceedings of the National Academy of Sciences. 88. 415-419.

253. Ruco, L.P., Ranalli, T., Marzullo, A., Bianco, P., Prat, M., Comoglio, P.M., Baroni, C.D. (1996) Expression of met protein in thyroid tumours. Journal of Pathology. 180. 266-270.

254. Rusciano, D., Lorenzoni, P., Burger, M.M. (1995) Expression of constitutively activated HGF/SF receptor (*c-met*) in B16 melanoma cells selected for enhanced liver colonisation. Oncogene. 11. 1979-1987.

255. Russell, W.E., McGowan, J.A., Bucher, N.L.R. (1984) Partial characterisation of an hepatocyte growth factor from rat platelets. Journal of Cellular Physiology. 119. 183-192.

256. Rygaard, K., Nakamura, T., Spang-Thomsen, M. (1993) Expression of proto-oncogenes *c-met* and *c-kit* and their ligands, hepatocyte growth factor/scatter factor and stem cell factor, in SCLC cell lines and xenografts. British Journal of Cancer. 67. 37-46.

257. Saccone, S., Narsimhan, R.P., Gaudino, G., Dalpra, L., Comoglio, P.M., Della Valle, G. (1992) Regional mapping of the human hepatocyte growth factor (HGF) scatter factor gene to chromosome 7q21.1. Genomics. 13. 912-914.

258. Sahin, A.A., Ro, J., Ro, J.Y., Blick, M.B., el-Naggar, A.K., Ordonez, N.G., Fritsche, H.A., Smith, T.L., Hortobagyi, G.N., Ayala, A.G. (1991) KI-67 immunostaining in node-negative stage I/II breast carcinoma. Significant correlation with prognosis. Cancer . 68. 549-557.

259. Sakakura, T. (1987). Mammary embryogenesis. The Mammary Gland: Development, Regulation and Function. Melvill, M., Daniel, C. New York: Plenum Press.

260. Sakakura, T. (1991) New aspects of stroma-parenchyma relations in mammary gland differentiation. International Review of Cytology. 125. 165-202.

261. Sanchez, M.P., Silos-Santiago, I., Frisen, J., He, B., Lira, S.A., Barbacid, M. (1996) Renal agenesis and the absence of enteric neurons in mice lacking GDNF. Nature. 382. 70-73.

262. Santos, O.F.P., Barros, E.J.G., Yang, X., Matsumoto, K., Nakamura, T., Park, M., Nigam, S.K. (1994) Involvement of HGF in kidney development. Developmental Biology. 163. 525-529.

263. Schmidt, C., Bladt, F., Goedecke, S., Brinkman, V., Zschiesche, W., Sharpe, M., Gherardi, E., Birchmeier, C. (1995) Scatter factor/hepatocyte growth factor is essential for liver development. Nature. 373. 699-702.

264. Schor, S.L., Schor, A.M., Winn, B., Rushton, G. (1982) The use of three dimensional gels for study of tumour cell invasion in vitro. International Journal of Cancer. 29. 57-62.

265. Schuchardt, A., D'Agati, V., Larsson-Blomberg, L., Costantini, F., Pachnis, V. (1994) Defects in kidney and enteric nervous system of mice lacking the tyrosine kinase receptor Ret. Nature. 367. 380-383.

266. Siegfried, J.M., Weissfeld, L.A., Singh Kaw, P., Weyant, R.J., Testa, J.R., Landreneau, R.J. (1997) Association of immunoreactive HGF with poor survival in resectable non-small cell lung cancer. Cancer Research. 57. 433-439.

267. Seslar, S., Nakamura, T., Byers, S. (1995) Tumor-stroma interactions and stromal cell density regulate hepatocyte growth factor protein levels: a role for transforming growth factor-beta activation. Endocrinology. 136. 1945-1953.

268. Seslar, S.P., Nakamura, T., Stephen, W.B. (1993) Regulation of fibroblast hepatocyte growth factor/scatter factor expression by human breast carcinoma cell lines and peptide growth factors. Cancer Research. 53. 1233-1238.

269. Shibamoto, S., Hayakawa, M., Hori, T., Oku, N., Miyazawa, K., Kitamura, N., Ito, F. (1992) HGF and TGF-beta stimulate both cell growth and migration of human gastric adenocarcinoma cells. Cell Structure and Function. 17. 185-190.

270. Shibamoto, S., Hayakawa, M., Takeuchi, K., Hori, T., Oku, N., Miyazawa, K., Kitamura, N., Takeichi, M., Ito, F. (1994) Tyrosine phosphorylation of beta -catenin and plakoglobin enhanced by hepatocyte growth factor and epidermal growth factor in human carcinoma cells. Cell. 1. 295-305.

271. Shima, N., Nagao, M., Ogaki, F., Tsuda, E., Murakami, A., Higashio, K. (1991) Tumor cytotoxic factor/HGF from human fibroblasts: cloning of its cDNA, purification and characterisation of recombinant protein. Biochemical and Biophysical Research Communications. 180. 1151-1158.

272. Shimomura, T., Denda, K., Kitamura, A., Kawaguchi, T., Kito, M., Kondo, J., Kagaya, S., Qin, L., Takata, H., Miyazawa, K., Kitamura, N. (1997) HGF activator inhibitor, a novel Kunitz-type serine protease inhibitor. Journal of Biological Chemistry. 272. 6370-6376.

273. Silvestrini, R., Daidone, M.G., Gasparini, G. (1985) Cell kinetics as a prognostic marker in node negative breast cancer. Cancer . 56. 1982-1987.

274. Singh-Kaw, P., Zarnegar, R., Siegfried, J.M. (1995) Stimulatory effects of HGF on normal and neoplastic human bronchial epithelial cells. American Journal of Physiology. 268. 1012-1020.

275. Sonnenberg, E., Meyer, D., Weidner, K.M., Birchmeier, C. (1993) Scatter factor/hepatocyte growth factor and its receptor, the *c-met* tyrosine kinase, can mediate a signal exchange between mesenchyme and epithelia during mouse development. Journal of Cell Biology. 123. 223-235.

276. Soriano, J.V., Pepper, M.S., Nakamura, T., Orci, L., Montesano, R. (1995) Hepatocyte growth factor stimulates extensive development of branching duct-like structures by cloned mammary gland epithelial cells. Journal of Cell Science. 108. 413-430.

277. Southern, E.M. (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. Journal of Molecular Biology. 98. 503-517.

278. Stern, C.D., Ireland, G.W., Herrick, S.E., Gherardi, E., Gray, J., Perryman, M., Stoker, M. (1990) Epithelial scatter factor and development of the chick embryonic axis. Development. 110. 1271-1284.

279. Stoker, M. (1984) Junctional competence in clones of mammary epithelial cells, and modulation by conditioned medium. Journal of Cellular Physiology. 121. 174-183.

280. Stoker, M., Gherardi, E., Perryman, M., Gray, J. (1987) Scatter factor is a fibroblast derived modulator of epithelial cell motility. Nature. 327. 239-241.

281. Stoker, M., Gherardi, M. (1991) Regulation of cell movement: the motogenic cytokines. Biochimica et Biophysica Acta - Reviews on Cancer. 1072. 81-102.

282. Stoker, M., Perryman, M. (1985) An epithelial scatter factor released by embryo fibroblasts. Journal of Cell Science. 77. 209-223.

283. Sunitha, I., Meighen, D.L., Hartman, D.P., Thompson, E.W., Byers, S.W., Avigan, M.I. (1994) Hepatocyte growth factor stimulates invasion across reconstituted basement membranes by a new human small intestinal cell line. Clinical and Experimental Metastasis. 12. 143-154.

284. Suzuki, K., Hayashi, N., Yamada, Y., Yoshihara, H., Miyamoto, Y., Ito, Y., Ito, T., Katayama, K., Sasaki, Y., Ito, A., Kisida, Y., Kashiwagi, T., Fusamoto, H., Kamada, T. (1994) Expression of the *c-met* protooncogene in human hepatocellular carcinoma. Hepatology. 20. 1231-1236.

285. Tajima, H., Matsumoto, K., Nakamura, T. (1992) Regulation of cell growth and motility by hepatocyte growth factor and receptor expression in several cell species. Experimental Cell Research. 202. 423-431.

286. Takada, N., Yano, Y., Matsuda, T., Otani, S., Osugi, H., Higashimoto, M., Kinoshita, H., Fukushima, S. (1995) Expression of immunoreactive hepatocyte growth factor in human esophageal squamous cell carcinomas. Cancer Letters. 97. 145-148.

287. Takahashi, S., Qian, J., Brown, J.A., Alacraz, A., Bostwick, D.G., Lieber, M.M., Jenkins, R.B. (1994) Potential markers of prostate cancer aggressiveness detected by in situ hybridisation in needle biopsies. Cancer Research. 54. 3574-3579.

288. Takahashi, S., Shan, A.L., Ritland, S.R., Delacey, K.A., Bostwick, D.G., Lieber, M.M., Thibodeau, S.N., Jenkins, R.B. (1995) Frequent loss of heterozygosity at 7q31.1 in primary prostate cancer is associated with tumour aggressiveness and progression. Cancer Research. 55. 4114-4119.

289. Takanami, I., Tanana, F., Hashizume, T., Kikuchi, K., Yamamoto, Y., Yamamoto, T., Kodaira, S. (1996) HGF and *c-met*/HGF receptor in pulmonary adenocarcinomas: an evaluation of their expression as prognostic markers. Oncology. 53. 392-397.

290. Takayama, H., LaRochelle, W.J., Sharp, R., Otsuka, T., Kriebel, P., Anver, M., Aaronson, S.A., Merlino, G. (1997) Diverse tumorigenesis associated with aberrant development in mice overexpressing HGF/SF. Proceedings of the National Academy of Sciences. 94. 701-706.

291. Takigawa, N., Segawa, Y., Maeda, Y., Takata, I., Fujimoto, N. (1997) Serum HGF/SF levels in small cell lung cancer patients. Lung Cancer. 17. 211-218.

292. Tamura, M., Arakaki, H., Tsubouchi, H., Takada, H., Daekuhara, Y. (1993) Enhancement of human hepatocyte growth factor production by interleukin-1α and -1β and tumour necrosis factor-α by fibroblasts in culture. Journal of Biological Chemistry. 268. 8140-8145.

293. Taniguchi, T., Kitamura, M., Arai, K., Iwasaki, Y., Yamamoto, Y., Igari, A., Toi, M. (1997) Increase in the circulating level of hepatocyte growth factor in gastric cancer patients. British Journal of Cancer. 75. 673-677.

294. Taniguchi, T., Toi, M., Inada, K., Imazawa, T., Yamamoto, Y., Tominaga, T. (1995) Serum concentrations of hepatocyte growth factor in breast cancer patients. Clinical Cancer Research. 1. 1031-1034.

295. Tannapfel, A., Yasui, W., Yokozaki, H., Wittekind, C., Tahara, E. (1994) Effect of hepatocyte growth factor on the expression of E- and P-cadherin in gastric carcinoma cell lines. Virchows Archiv. 425. 139-144.

296. Tannock, I.F. (1968) The relation between cell proliferation and the vascular system in a transplanted mouse mammary tumour. British Journal of Cancer. 22. 258-273.

297. Tempest, P.R., Stratton, M.R., Cooper, C.S. (1988) Structure of the met protein and variation of met protein kinase activity among human tumour cell lines. British Journal of Cancer. 58. 3-7.

298. Toi, M., Hoshina, S., Takayanagi, T., Tominaga, T. (1994) Association of vascular endothelial growth factor expression with tumour angiogenesis and early relapse in primary breast cancer. Japanese Journal of Cancer Research. 85. 1045-1049.

299. Toi, M., Inada, K., Suzuki, H., Tominaga, T. (1995) Tumour angiogenesis in breast cancer: its importance as a prognostic indicator and the association with vascular endothelial growth factor expression. British Journal of Cancer Research and Treatment. 36. 193-204.

300. Toi, M., Kondo, S., Suzuki, H., Yamamoto, Y., Inada, K., Imazawa, T., Taniguchi, T., Tominaga, T. (1996) Quantitative analysis of vascular endothelial growth factor in primary breast cancer. Cancer . 77. 1101-1106.

301. Toi, M., Taniguchi, T., Ueno, T., Asano, M., Funata, N., Sekiguchi, K., Iwanari, H., Tominaga, T. (1998) Significance of circulating HGF level as a prognostic indicator in primary breast cancer. Clinical Cancer Research. 4. 659-664.

302. Tripathy, D., Benz, C. (1994) Growth factors and their receptors. Haematology/Oncology Clinics of North America. 8. 29-49.

303. Tsarfaty, I., Resau, J.H., Shen, R.L., Keydar, I., Faletto, D.L., Vande Woude, G.F. (1992) The MET protooncogene receptor and lumen formation. Science. 257. 1258-1261.

304. Tsarfaty, I., Rong, S., Resau, J.H., Rulong, S., Da Silva, P.P., Vande Woude, G.F. (1994) The Met proto-oncogene mesenchymal to epithelial cell conversion. Science. 263. 98-101.

305. Tsubouchi, H., Hirono, S., Gohda, E., Nakayama, H. Takahashi, K., Sakiyama, O., Miyazaki, H., Sugihara, J., Tomita, E., Muto, Y. (1989) Clinical significance of

human hepatocyte growth factor in blood from patients with fulminant hepatic failure. Hepatology. 9. 875-881.

306. Tsuda, H., Iwase, T., Matsumoto, K., Ito, M., Hirono, I., Nishida, Y., Yamamoto, M., Tatematsu, M., Nakamura, T. (1992) Immunohistochemical localisation of hepatocyte growth factor protein in pancreatic islet A-cells of man and rats. Japanese Journal of Cancer Research. 83. 1262-1266.

307. Tuck, A.B., Park, M., Sterns, E.E., Boag, A., Elliott, B.E. (1996) Coexpression of HGF and receptor (Met) in human breast cancer. American Journal of Pathology. 148. 225-232.

308. Uchiyama, A., Essner, R., Doi, F., Nguyen, T., Ramming, K.P., Nakamura, T., Morton, D.L., Hoon, D.S. (1996) Interleukin-4 inhibits HGF-induced invasion and migration of colon carcinomas. Journal of Cellular Biochemistry. 62. 443-453.

309. Uehara, Y., Kitamura, N. (1992) Expression of human hepatocyte growth factor/scatter factor cDNA in MDCK epithelial cells influences cell morphology, motility and anchorage independent growth. Journal of Cell Biology. 117. 889-894.

310. Uehara, Y., Minowa, O., Mori, C., Shiota, K., Kuno, J., Noda, T., Kitamura, N. (1995) Placental defects and embryonic lethality in mice lacking hepatocyte growth factor/scatter factor. Nature. 373. 702-705.

311. Van de Hooff, A. (1991) The role of stromal cells in tumour metastasis: a new link. Cancer Cells. 3. 186-187.

312. Van den Hoff, A. (1998) Stromal involvement in malignant growth. Advances in Cancer Research. 50. 159-196.

313. Vande Woude, G.F., Jeffers, M., Cortner, J., Alvord, G., Tsarfaty, I., Resau, J. (1997) Met-HGF/SF: tumorigenesis, invasion and metastasis. Ciba Foundation Symposium. 212. 119-132.

314. Vartanian, R.K., Weidner, N. (1994) Correlation of intratumoral endothelial cell proliferation with microvessel density (tumor angiogenesis) and tumor proliferation in breast carcinoma. American Journal of Pathology. 144. 1188-1194.

315. Visscher, D.W., Wallis, T.L., Crissman, J.D. (1996) Evaluation of chromosome aneuploidy in tissue sections of preinvasive breast carcinomas using interphase cytogenetics. Cancer . 77. 315-320.

316. Walker, R.A., Jones, J.L., Chappell, S., Walsh, T., Shaw, J.A. (1997) Molecular pathology of breast cancer and its application to clinical management. Cancer Metastasis Reviews. 16. 5-27.

317. Wang, Y., Selden, A.C., Morgan, N., Stamp, G.W.H., Hodgson, H.J.F. (1994) Hepatocyte growth factor/scatter factor expression in human mammary epithelium. American Journal of Pathology. 144. 675-682.

318. Wang, Y., Selden, C., Farnaud, S., Calnan, D., Hodgson, H.J.F. (1994) Hepatocyte growth factor (HGF/SF) is expressed in human epithelial cells during embryonic development; studies by in situ hybridisation and Northern blot analysis. Journal of Anatomy. 185. 543-551.

319. Weidner, K.M., Arakaki, N., Hartmann, G., Vandekerckhove, J., Weingart, S., Reider, H., Fonatsch, C., Tsubouchi, H., Hishida, T., Daikuhara, Y., Birchmeier, W. (1991) Evidence for the identity of human scatter factor and human hepatocyte growth factor. Proceedings of the National Academy of Sciences. 88. 7001-7005.

320. Weidner, K.M., Behrens, J., Vandekerckhove, J., Birchmeier, W. (1990) Scatter factor: molecular characteristics and effect on the invasiveness of epithelial cells. Journal of Cell Biology. 111. 2097-2108.

321. Weidner, K.M., Sachs, M., Birchmeier, W. (1993) The Met receptor tyrosine kinase transduces motility, proliferation, and morphogenic signals of scatter factor/hepatocyte growth factor in epithelial cells. Journal of Cell Biology. 121. 145-154.

322. Weidner, K.M., Sachs, M., Riethmacher, D., Birchmeier, W. (1995) Mutation of juxtamembrane tyrosine residue 1001 suppresses loss-of-function mutations of the met receptor in epithelial cells. Proceedings of the National Academy of Sciences. 92. 2597-2601.

323. Weidner, N. (1993) Tumor angiogenesis: Review of current applications in tumor prognostication. Seminars in Diagnostic Pathology. 10. 302-313.

324. Weidner, N. (1998) Tumoural vascularity as a prognostic factor in cancer patients: The evidence continues to grow. Journal of Pathology. 184. 119-122.

325. Weidner, N., Semple, J.P., Welch, W.R., Folkman, J. (1991) Tumor angiogenesis and metastasis-correlation in invasive breast carcinoma. The New England Journal of Medicine. 324. 1-8.

326. Weimar, I.S., de Jong, D., Muller, E.J., Nakamura, T., van Gorp, J.M., de Gast, G.C., Gerritsen, W.R. (1997) HGF/SF promotes adhesion of lymphoma cells to extracellular matrix molecules via alpha (4)beta(1) and alpha(5)beta(1) integrins. Blood. 89. 990-1000.

327. Wintzer, H.O., Zipfel, I., Schulte-Monting, J. (1991) KI-67 immunostaining in human breast tumours and its relationship to prognosis. Cancer . 67. 421-428.

328. Wolf, H.K., Zarnegar, R., Michalopoulos, G.K. (1991) Localisation of hepatocyte growth factor in human and rat tissues: an immunohistochemical study. Hepatology. 14. 488-494.

329. Wolf, H.K., Zarnegar, R., Oliver, L., Michalopoulos, G.K. (1991) Hepatocyte growth factor in human placenta and trophoblastic disease. American Journal of Pathology. 138. 1035-1043.

330. Xiao, S., Geng, J.S., Feng, X.L., Liu, X.Q., Liu, Q.Z., Li, P. (1992) Cytogenetic studies of eight primary gastric cancers. Cancer Genetics and Cytogenetics. 58. 79-84.

331. Yamashita, J.I., Ogawa, M., Beppu, T. (1993) Immunoreactive hepatocyte growth factor is present in tissue extracts from human breast cancer but not in conditioned medium of human breast cancer cell lines. Research Communications in Chemical Pathology and Pharmacology. 82. 249-252.

332. Yamashita, J.I., Ogawa, M., Yamashita, S.I., Nomura, K., Kuramoto, M., Saishoji, T., Shin, S. (1994) Immunoreactive hepatocyte growth factor is a strong and independent predictor of recurrence and survival in human breast cancer. Cancer Research. 54. 1630-1633.

333. Yang, J., Guzman, R., Richards, J., Jentoft, V., DeVault, M.R., Wellings, S.R., Nandi, S. (1980) Primary culture of human mammary epithelial cells embedded in collagen gels. Journal of the National Cancer Institute. 65. 337-343.

334. Yang, Y., Spitzer, E., Meyer, D., Sachs, M., Niemann, C., Hartmann, G., Weidner, K.M., Birchmeier, C., Birchmeier, W. (1995) Sequential requirement of hepatocyte growth factor and neuregulin in the morphogenesis and differentiation of mammary gland. Journal of Cell Biology. 131. 215-226.

335. Yao, Y., Jin, L., Fuchs, A., Joseph, A., Hastings, H.M., Goldberg, I.D., Rosen, E.M. (1996) Scatter factor protein levels in human breast cancers. American Journal of Pathology. 149. 1707-1717.

336. Yaremko, M.L., Recant, W.M., Westbrook, C.A. (1995) Loss of heterozygosity from the short arm of chromosome 8 is an early event in breast cancers. Genes Chromosomes and Cancer. 13. 186-191.

337. Yoshinaga, Y., Fujita, S., Gotoh, M., Nakamura, T., Kikuchi, M., Hirohashi, S. (1992) Human lung cancer cell line producing hepatocyte growth factor/scatter factor. Japanese Journal of Cancer Research. 83. 1257-1261.

338. Yoshinaga, Y., Matsuno, Y., Fujita, S., Nakamura, T., Kikuchi, M., Shimosato, Y., Hirohashi, S. (1993) Immunohistochemical detection of hepatocyte growth

factor/scatter factor in human cancerous and inflammatory lesions of various organs. Japanese Journal of Cancer Research. 84. 1150-1158.

339. Zarnegar, R., Michalopoulos, G. (1989) Purification and biological characteristion of human hepatopoetin A, a polypeptide growth factor for hepatocytes. Cancer Research. 49. 3314-3320.

340. Zarnegar, R., Muga, S., Enghild, J., Michalopoulos, G. (1989) NH2 terminal amino acid sequence of rabbit haemopoetin A, a heparin binding polypeptide growth factor for hepatocytes. Biochemical and Biophysical Research Communications. 163. 1370-1376.

341. Zarnegar, R., Muga, S., Rahija, R., Michalopoulos, G. (1990) Tissue distribution of haemopoetin A: a heparin binding polypeptide growth factor for hepatocytes. Proceedings of the National Academy of Sciences. 87. 1252-1256.

342. Zenklusen, J.C., Bieche, I., Lidereau, R., Conti, C.J. (1994) (C-A)n microsatellite repeat D7s522 is the most commonly deleted region in human primary breast cancer. Proceedings of the National Academy of Sciences. 91. 12155-12158.

343. Zenklusen, J.C., Oshimura, M., Barrett, J.C., Conti, C.J. (1994) Inhibition of tumorigenicity of a murine squamous carcinoma cell line by a putative tumour suppressor gene on human chromosome 7. Oncogene. 9. 2817-2825.

344. Zenklusen, J.C., Thompson, J.C., Klein-Szanto, A.J.P., Conti, C.J. (1995) Frequent loss of heterozygosity in human primary squamous cell and colon carcinomas at 7q31.1: evidence for a broad range tumour suppressor gene. Cancer Research. 55. 1347-1350.

345. Zenklusen, J.C., Thompson, J.C., Troncosa, P., Kagan, J., Conti, C.J. (1994) Loss of heterozygosity in human primary prostate carcinomas: a possible tumour suppressor gene at 7q31.1. Cancer Research. 54. 6370-6373.

346. Zenklusen, J.C., Weitzel, J.N., Ball, H.G., Conti, C.J. (1995) Allelic loss at 7q31.1 in human primary ovarian carcinomas suggests the existence of a tumour suppressor gene. Oncogene. 11. 359-363.

347. Zhang, J.S., Nelson, M., Mviver, B., Hay, I.D., Goellner, J.R., Grant, C.S., Eberhardt, N.L., Smith, D.I. (1998) Differential loss of heterozygosity at 7q31.2 in follicular and papillary thyroid tumours. Oncogene. 17. 789-793.

348. Zhu, H., Naujokas, M.A., Fixman, E.D., Torossian, K., Park, M. (1994) Tyrosine 1356 in the carboxyl-terminal tail of the HGF/SF receptor is essential for the transduction of signals for cell motility and morphogenesis. Journal of Biological Chemistry. 269. 29943-29948.

349. Zhu, H., Naujokas, M.A., Park, M. (1994) Receptor chimeras indicate that the met tyrosine kinase mediates the motility and morphogenic responses of hepatocyte growth/scatter factor. Cell Growth and Differentiation. 5. 359-366.

Appendix 1

Clinicopathological variables, disease-free, overall survival, *c-met* expression and HGF/SF levels in 73 patients with breast cancer.

Patient Number	Age (years)	Size (mm)	Grade	Nodes	ER	LI	RFS (months)	OS (months)	*c-met*	HGF/SF
478	52	40	2	P	N	N	7	31	P	677
248	69	45	1	P	P	N	43	60	P	440
474	33	40	3	P	P	P	49	59	P	132
275	61	50	2	N	N	N	5	12	P	729
379	43	50	2	P	P	P	19	47	P	148
533	54	22	3	P	?	N	4	7	P	915
437	55	60	2	P	N	N	31	36	P	465
505	41	23	3	P	P	P	14	21	P	530
529	38	15	3	N	N	N	15	25	P	427
512	60	20	2	P	P	P	10	18	P	513
503	56	20	2	N	P	P	36	56	P	621
400	63	55	3	P	N	N	18	24	P	350
296	50	35	3	P	N	P	10	30	P	595
213	44	60	2	P	P	N	36	40	N	?
300	44	60	2	P	N	N	9	23	N	441
145	74	40	3	N	?	N	19	19	N	252
272	74	25	3	N	N	N	18	30	N	603
446	74	55	2	P	N	N	12	15	N	330
475	66	35	3	P	P	N	4	29	N	1604
293	57	60	2	P	N	P	7	13	N	163
425	67	50	2	N	P	N	32	34	N	995
292	73	25	2	N	?	N	7	7	N	?
261	62	30	3	P	N	N	8	20	N	552
377	66	20	3	P	N	P	5	19	N	491
373	64	25	2	P	P	P	35	48	N	1045
220	63	60	1	NDiss	N	N	34	>60	P	320
522	82	30	2	P	P	P	42	>60	P	798
531	78	15	2	N	?	N	49	>60	P	462
372	56	40	Medullary	N	N	N	>60	>60	P	420
289	67	25	Mucoid	P	P	N	>60	>60	P	255
310	52	20	3	P	N	P	>60	>60	P	184
547	57	30	2	P	?	P	>60	>60	P	135
511	60	12	1	N	P	N	>60	>60	P	393
256	49	25	2	N	N	N	>60	>60	P	331
206	64	30	Lobular	N	P	N	>60	>60	P	58
294	73	25	2	P	N	P	>60	>60	P	168
578	49	40	3	N	?	N	>60	>60	P	297
489	50	20	2	P	?	P	>60	>60	P	78
455	63	30	3	NDiss	P	P	>60	>60	N	120
257	49	60	3	P	N	N	>60	>60	N	231
560	55	45	3	P	?	N	>60	>60	N	310
165	41	25	2	N	N	N	>60	>60	N	832

Patient Number	Age (years)	Size (mm)	Grade	Nodes	ER	LI	RFS (months)	OS (months)	*c-met*	HGF/SF
330	55	25	2	N	P	N	>60	>60	N	100
407	54	30	Lobular	NDiss	N	N	>60	>60	N	243
477	66	40	3	P	P	N	>60	>60	N	396
561	71	25	3	P	?	P	>60	>60	N	60
396	62	30	3	N	N	N	>60	>60	N	406
319	65	30	Lobular	N	N	N	>60	>60	N	374
217	42	40	3	N	N	N	>60	>60	N	60
383	65	20	2	N	P	P	>60	>60	N	324
260	78	50	2	N	P	N	>60	>60	N	315
451	73	10	3	N	N	N	>60	>60	N	129
374	50	25	3	N	P	P	>60	>60	N	911
154	36	20	3	N	?	N	>60	>60	N	62.4
516	63	20	2	N	P	N	>60	>60	N	383
515	45	25	2	NDiss	P	N	>60	>60	N	456
96	74	20	1	N	P	N	>60	>60	N	66
494	64	40	3	P	?	N	>60	>60	N	319
313	39	28	2	N	N	N	>60	>60	N	233
517	64	20	2	P	P	N	>60	>60	N	345
334	54	30	3	P	N	P	>60	>60	N	370
171	35	20	2	P	P	N	>60	>60	N	95
241	62	10	1	N	P	N	>60	>60	N	152
410	36	60	3	N	P	P	>60	>60	N	620
462	43	20	Lobular	P	P	N	>60	>60	N	84
442	50	20	2	N	N	N	>60	>60	N	475
436	62	5	1	N	N	N	>60	>60	N	185
306	42	40	1	P	N	N	>60	>60	N	84
433	72	20	1	P	P	N	>60	>60	N	473
137	46	55	Mucoid	N	N	N	>60	>60	N	510
417	40	15	3	P	N	N	>60	>60	N	588
565	64	30	2	N	?	N	>60	>60	N	279
151	68	20	2	N	P	P	>60	>60	N	558

Key

N	negative
P	positive
NDiss	not dissected
ER	oestrogen receptor status
LI	lymphatic invasion
RFS	relapse-free survival
OS	overall survival
HGF/SF	ng/100mg total protein.

C-met and HGF/SF levels in tumour-free breast tissue.

Patient number	***c-met***	**HGF/SF**
561N	P	90
533N	P	88
RM	P	213
319N	P	101
560N	P	114
213N	P	170
424N	P	100
063N	P	162
151N	P	66
522N	P	200

Key

RM	reduction mammoplasty
P	positive
HGF/SF	ng/100mg total protein.

Appendix 2

Multivariate Analyses (performed by Dr. I C. McKay, University Dept. Pathology, Western Infirmary, Glasgow)

1. Multiple linear regression

The following were used as potential prognostic indicators to relapse-free survival (RFS) and overall survival:

- HGF in tumour / ng per l00mg protein
- MET in tumour
- Diameter of tumour / mm
- Age / yrs
- Metastases in lymph nodes
- Oestrogen receptors (ER)
- Invasion of lymphatics (LI)
- Histological grade

By stepwise removal of non-significant factors from the model, we were left with the following variables in our regression equation:

Variable	Coefficient	SE of coeff	T	p
HGF	-0.032512	0.008833	-3.681	0.0006
MET	-12.841323	5.553414	-2.312	0.0254
Nodes	-13.142986	5.303057	-2.478	0.0170
ER	11.448547	5.226806	2.190	0.0337
(Constant)	63.017702	5.771831	10.918	0.0000

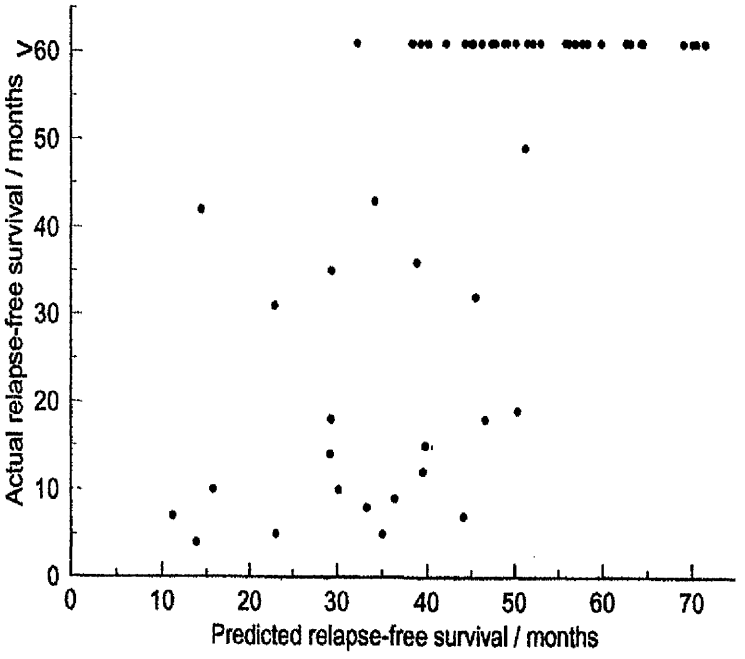
Thus, in addition to the variables already found to be of probable prognostic value, it seems that the presence of oestrogen receptors, when considered along with other factors, may be of some prognostic value. To allow for the interaction (both biochemical and statistical) between HGF and MET, a non-linear regression model was used in which the product MET x HGF is introduced, in this model the predicted value of RFS is calculated from a formula of the form

$$RFS = (a \times MET \times HGF) + (b \times ER) + (c \times nodes) + (d \times HGF) + (e \times MET) + k,$$

where MET, HGF, ER, nodes are all coded 0 for negative and 1 for positive, and the HGF is measured in ng per l00mg protein. The parameters a, b, c, d, k were all adjusted to make the prediction as accurate as possible, as judged by the sum of the squares of the errors in predicted RFS. In this model it was found that the term containing MET by itself (without HGF) became statistically non-significant, the effect of MET being entirely attributable to its interaction with HGF. After removing MET from the model and adjusting the coefficients to give the best predictions, the optimised coefficients were found to have the following values.

Parameter	Estimate	Asymptomatic Std. error	Asymptomatic 95% Confidence Interval	
			Lower	Upper
a	-0.027523651	0.010252666	-0.048106736	-0.006940565
b	9.867950158	4.650936421	0.530805683	19.205094634
c	-14.77398629	4.671878632	-24.15317401	-5.394798574
d	-0.027909886	0.008227353	-0.044426987	-0.011392786
k	63.452290577	5.072409907	53.269002771	73.636678382

The extent to which this model could predict RFS is illustrated in the following graph.



2. Linear discriminant analysis

This technique consists of calculating a linear function (the 'discriminant function') of the potential prognostic indicators and optimising it until it gives the best prediction of which patients will have 5 years or more recurrence-free survival, and which patients will not. In the process, the prognostic indicators are tested to see if they are making a statistically significant contribution to the accuracy of the prognosis, and those that are not are removed from the model.

Technical details:

Method:	Wilks' lambda	
Criteria:	F value for entry of a factor:	3.84
	F values for removal of a factor:	2.71

Prior probabilities computed from group sizes.
Missing values replaced with mean values.
Discriminant function based on the within-groups covariance matrix.

Initially the following variables were considered for inclusion in the model:
AGE, SIZE, GRADE, NODES, ER, LI, MET, HGF

Only three prognostic indicators satisfied the criterion for inclusion in the model, and none thereafter satisfied the criterion for removal from the model. These indicators that appeared to contribute significantly to the prognosis were as follows.

Variable added Step to the model	Wilks' Lambda	P value	Label
1 MET	.77957	.0006	MET in tumour
2 HGF	.61652	.0000	HGF in tumour / ng per l00mg protein
3 NODES	.53069	.0000	Metastases in lymph nodes

After these variables had been included in the model the next most significant was the size of the tumour/mm but the significance was not nearly high enough to justify its inclusion (P =0.317).

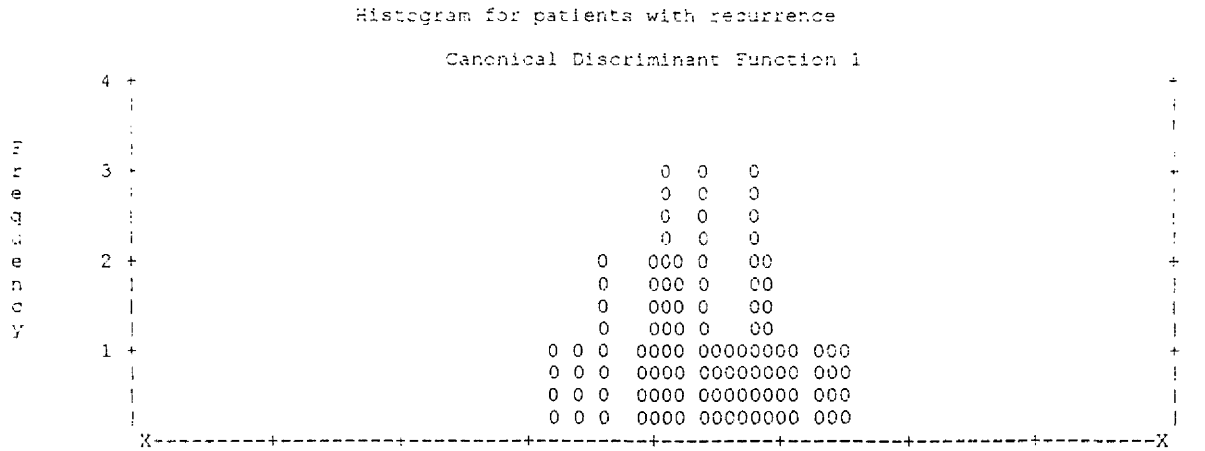
Unstandardised canonical discriminant function coefficients

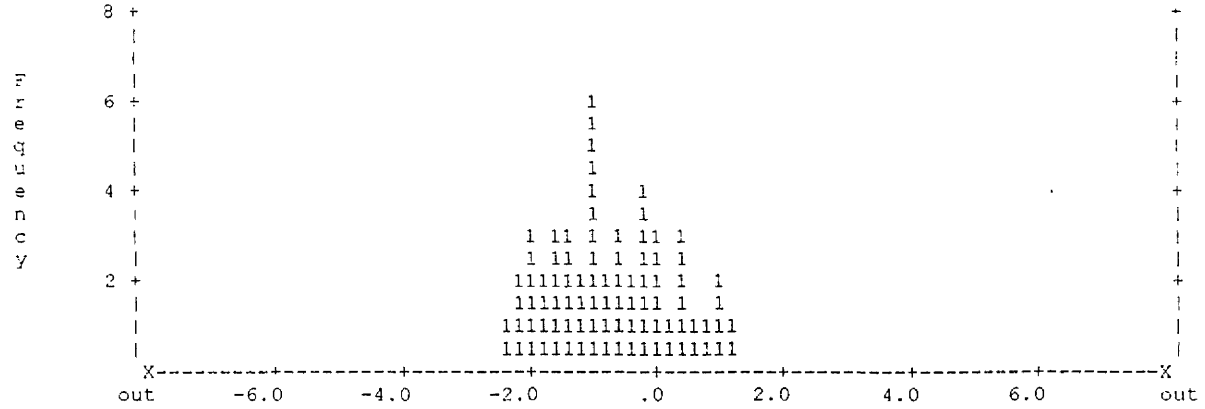
NODES	1.1896617
MET	1.6425366
HGF	2.59976921 E-03
(Constant)	-2.3451422

Thus, the recipe for prediction is as follows: calculate a score by multiplying the HGF level (in ng per l00mg protein) by 2.6 x 10^{-3}; add 1.19 if there are metastases in the nodes; add 1.64 if the tumour is MET positive. Subtract 2.345 and call the answer your discriminant function. If the answer is less than 0.2 you predict 5 years of recurrence-free survival. The distribution of the patients over the range of values of this discriminant function is shown on the following rough histograms. It can be seen that there is a fair overlap between the two groups (recurrence and no recurrence).

Symbols used in plots:

0 recurrence in 5 years 1 5 years' recurrence-free survival



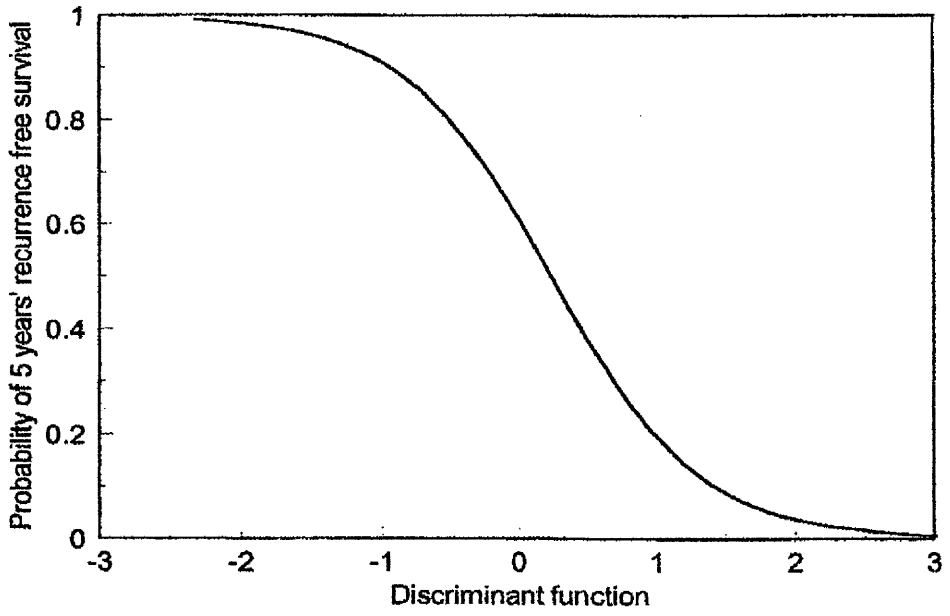


Classification of results:

	No. of cases	Predicted group membership	
Actual group		Recurrence	No recurrence
Recurrence	28	20 (71.4%)	8 (28.6%)
No recurrence	45	8 (17.8%)	37 (82.2%)

Percent of "grouped" cases correctly classified: 78.08%.

Alternatively, the discriminant function can be used to estimate the probability of recurrence-free survival as follows:



Probability of 5 years' recurrence-free survival for various values of the linear discriminant function.

3. Non-linear discriminant analysis

This was carried out using the same criteria as in the linear discriminant analysis, but included among the prognostic indicators was the product obtained by multiplying two of them together, namely MET x HGF.

The technique consists of calculating an algebraic function (the 'discriminant function') of the potential prognostic indicators and optimising it until it gives the best prediction of which patients will have 5 years or more recurrence-free survival, and which patients will not. In the process, the prognostic indicators are tested to see if they are making a statistically significant contribution to the accuracy of the prognosis, and those that are not are removed from the model.

Method: Wilks' lambda
Criteria: F value for entry of a factor: 3.84
F values for removal of a factor: 2.71
Prior probabilities computed from group sizes.
Missing values replaced with mean values.
Discriminant function based on the within-groups covariance matrix.

Initially the following variables were considered for inclusion in the model:

AGE, SIZE, GRADE, NODES, ER, LI, MET, HGF, MET x HGF

In this analysis one of the interesting findings was that the effect of MET by itself (independent of HGF) became statistically non-significant; i.e. there was no evidence that MET had any other effect than to alter the prognostic weight that needed to be given to the HGF assay. It had no apparent influence independent of HGF. The other interesting aspect of this model was that it gave more accurate prognoses than the linear model, despite the fact that the discriminant function still contained only three significant terms.

Only three prognostic indicators satisfied the criterion for inclusion in the model, and none thereafter satisfied the criterion for removal from the model. These indicators that appeared to contribute significantly to the prognosis were as follows.

Variable added Step to the model	**Wilks' Lambda**	**p value**	**Label**
1 MET x HGF	.72341	.0001	
2 NODES	.61155	.0000	Metastases in lymph nodes
3 HGF	.51920	.0000	HGF in tumour / ng per l00mg protein

After these variables had been included in the model the next most significant was the size of the tumour/mm but the significance was not nearly high enough to justify its inclusion (p =0.386).

Unstandardized canonical discriminant function coefficients

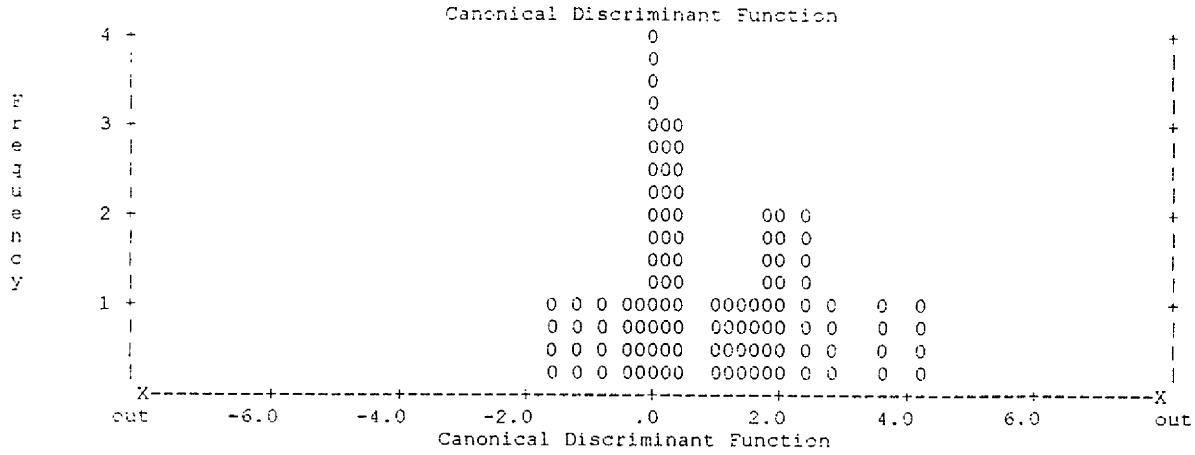
NODES	1.3166844
HGF	2.05971933E-03
MET x HGF	3.37683212E-03
(Constant)	-2.1241668

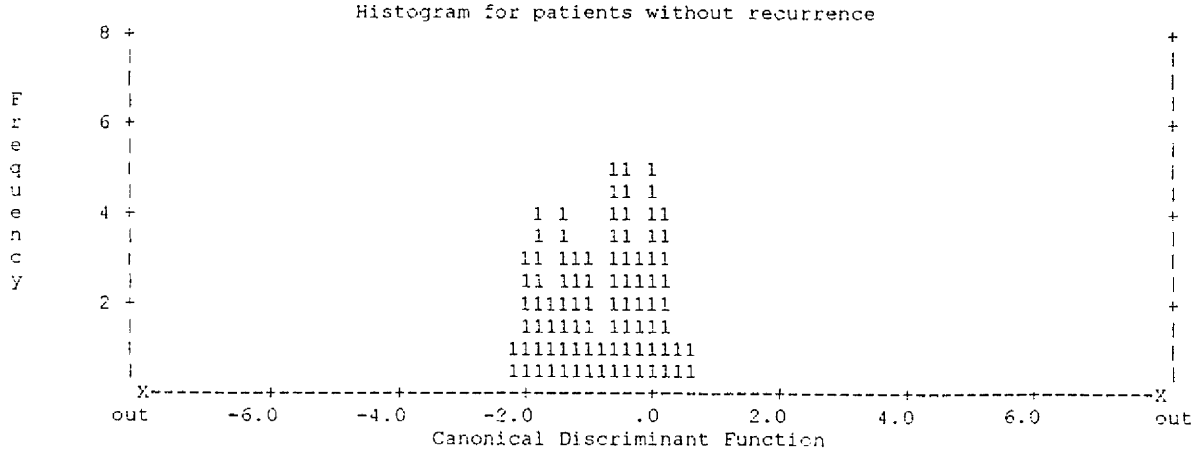
Thus, the recipe for prediction is as follows: calculate a score by multiplying the HGF level (in ng of HGF per l00mg of tumour protein) by 0.00206 if the tumour is MET negative, or by 0.00544 if the tumour is MET positive; add 1.32 if there are metastases in the nodes. Subtract 2.124 and call the answer your discriminant function. If the answer is less than 0.263 you predict 5 years of recurrence-free survival.

The distribution of the patients over the range of values of this discriminant function is shown on the following rough histograms. It can be seen that there is fair overlap between the two groups (recurrence and no recurrence).

Symbols used in plots:

0 recurrence within 5 years 1 5 years' recurrence-free survival.



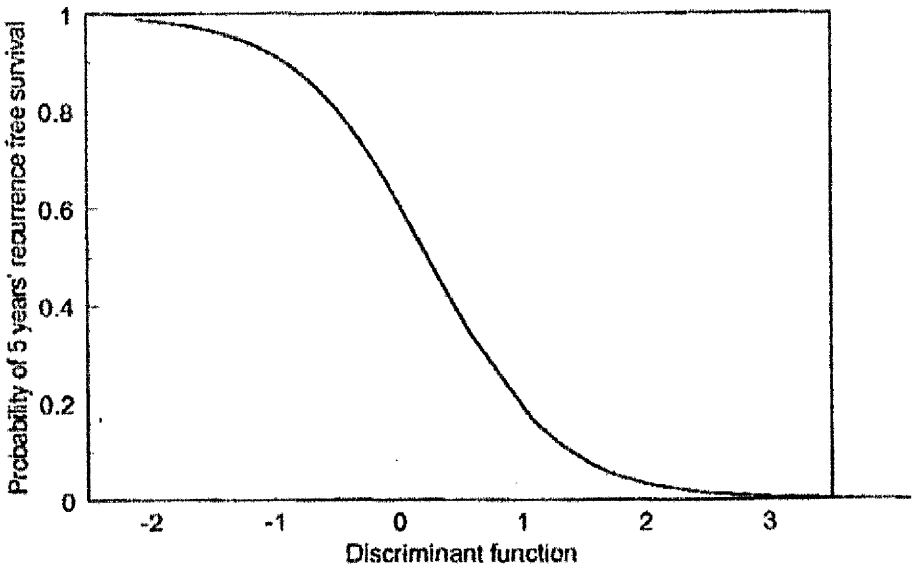


Classification of results:

	No. of cases	Predicted group membership	
Actual group		Recurrence	No recurrence
Recurrence	28	16 (57.1%)	12 (42.9%)
No recurrence	45	2 (4.4%)	43 (95.6%)

Percent of "grouped" cases correctly classified: 80.82%

An alternative to the prognosis is to use the value of the discriminant function to estimate the probability of 5 years' recurrence-free survival using the following graph.



4. Analysis of variance and covariance

In this technique, as with the linear regression analysis, an attempt is made to predict the recurrence-free survival from the available prognostic indicators, but the continuously-variable factors like age, size/mm, HGF level (the covariates) are treated differently from the discretely-valued factors, namely grade, nodes, ER, LI, MET. Each factor is tested for the statistical significance of its relationship with the 'dependent variable', namely recurrence-free survival.

In the first model used, all the above factors were included. This was done using the Minitab command
mtb > glm c9=c2 c4 c5 c6 c7 c8 c11 c12;
subc> cova c2 c4 c12.

The result indicated that the predictive value of most of the factors were not statistically significant, as seen by the P values in the analysis of variance table below.

Analysis of Variance for recurrence-free survival

Source	**DF**	**Seq SS**	**Adj SS**	**Adj MS**	**F**	**p**
Age	1	47.2	197.7	197.7	0.60	0.444
Size/mm	1	3545.5	1082.5	1082.5	3.28	0.078
Grade	2	1267.1	145.5	72.7	0.22	0.803
Nodes	1	2027.0	1136.9	1136.9	3.44	0.071
ER	1	449.9	835.2	835.2	2.53	0.119
LI	1	114.9	0.1	0.1	0.00	0.989
MET	1	1751.5	1500.6	1500.6	4.54	0.039
HGF	1	2758.8	2758.8	2758.8	8.35	0.006
Error	41	13538.3	13538.3	330.2		
Total	50	25500.2				

The clearly insignificant terms were then sequentially removed from the statistical model, and first-

order interaction terms were introduced, wherever this was found to be justifiable as judged by a significant reduction in the residual sum of squares. On introduction of the MET*HGF interaction term, MET by itself became insignificant, but it was retained in order to keep the model hierarchical. This gave the following results.

Analysis of Variance for recurrence-free survival

Source	DF	Seq SS	Adj SS	Adj MS	F	p
Size/mm	1	2550.9	1500.5	1500.5	4.52	0.037
MET	1	1730.8	533.3	533.3	1.61	0.210
HGF	1	4548.3	6724.7	6724.7	20.24	0.000
MET*HGF	1	2155.3	2271.0	2271.0	6.84	0.011
Nodes	1	1277.6	1277.6	1277.6	3.85	0.054
Error	63	20930.2	20930.2	332.2		
Total	68	33193.0				

It can be seen that the factors found to be of most significance as predictors of RFS were HGF and the interaction between HGF and MET. When the interaction term was included in the model, MET by itself did not have a statistically significant effect. In other words, the effects of MET could be modeled adequately by the hypothesis that it simply increased the influence of HGF, but did not have any other influence of its own.

The presence of metastases in the nodes, and the size of the tumour in mm, were of marginal significance, and a closer study was made of their prognostic value, given that MET and HGF effects were already being considered. The removal of either size or nodes from the statistical model tended to make the other more significant. The most likely explanation for this is that the information contained in them overlaps substantially. In other words, the size of the tumour may be of prognostic value mainly because it is associated with a greater risk of metastases, and this greater risk is also reflected in the lymph node involvement The effect of removing either nodes or size from the prognostic model is illustrated below.

Analysis of Variance for recurrence-free survival with nodes excluded

Source	DF	Seq SS	Adj SS	Adj MS	F	p
MET	1	2100.9	308.9	308.9	0.94	0.337
HGF	1	4959.3	6853.8	6853.8	20.77	0.000
MET*HGF	1	2004.2	2141.2	2141.2	6.49	0.013
Size/mm	1	2557.1	2557.1	2557.1	7.75	0.007
Error	68	22440.7	22440.7	330.0		
Total	72	34062.2				

Analysis of Variance for recurrence-free survival with size excluded

Source	DF	Seq SS	Adj SS	Adj MS	F	p
MET	1	1674.0	581.2	581.2	1.66	0.202
HGF	1	4846.6	6911.3	6911.3	19.72	0.000
MET*HGF	1	2083.3	2244.9	2244.9	6.41	0.014
Nodes	1	2158.2	2158.2	2158.2	6.16	0.016
Error	64	22430.7	22430.7	350.5		
Total	68	33193.0				

Conclusions

The analysis of variance and the discriminant analysis lead to much the same conclusions.

1. The prognostic factors that were found to contribute significantly to the prediction of relapse-free survival were HGF and MET. In some statistical models there was also a strong indication that the presence of metastases in the lymph nodes, and the size of the tumour (diameter in mm) was of prognostic significance. In only one model was there any indication that the presence of estrogen receptors might be of some prognostic significance.
2. There was no evidence in any of our analyses that the age of the patient, the histological grade of the tumour or invasion of the lymphatics were of any prognostic significance.
3. There was a significant statistical interaction between MET and HGF, as judged by discriminant analysis and by analysis of variance and covariance. In other words, the combined effect of the two was greater than the sum of their individual effects.
4. When this interaction was included in our statistical models, either in the discriminant analysis or in the analysis of variance and covariance, the direct effect of MET became statistically insignificant. In other words, the effects of MET could be attributed entirely to the fact that its presence enhanced the influence of HGF. There was no evidence that MET had any effect independent of HGF.
5. The converse was not true: in other words, HGF appeared to be of prognostic significance even when MET was apparently absent from the tumour, as judged by our assay. However, it is possible that with a more sensitive MET assay we might find that some of our apparently MET-negative tumours do in fact contain some MET-positive cells. It is still possible therefore that the influence of HGF may depend on the presence of MET.
6. The most significant of all the prognostic indicators was HGF.

Appendix 3

HGF/SF levels, % tumour vascular volume (%TVV), microvessel density (MVD) and KI-67 index in 73 patients with breast cancer.

Number	MVD	%TVV	KI-67%	HGF/SF
96	37	4.1	5	66
137	69	4.7	5	510
145	41	3.9	20.5	252
151	97	10.3	22	558
154	39	3.8	5	62.4
165	111	10.2	32	832
171	76	5.8	7	95
206	89	6.6	20	58
213	115	15	5	?
217	123	9.8	5	60
220	119	7.1	5	320
241	43	2.9	4	152
248	74	5.8	17	440
256	49	3.4	5	331
257	75	3.4	24	231
260	85	3.4	18	315
261	83	7.7	7	552
272	64	6.5	28	603
275	43	4	26	729
289	63	5.9	12	255
292	82	2	25	?
293	48	5	25	163
294	103	6.1	24	168
296	109	9.2	18	595
300	63	4.4	28	441
306	57	5.3	13	84
310	37	3.1	34	184
313	119	5.2	59	233
319	121	11.1	23	374
330	74	5.1	26	100
334	30	5.4	16	370
372	55	6.5	16	420
373	41	8.9	46	1045
374	83	3.3	6	911
377	72	9.4	30	491
379	44	3.8	4	148
383	45	5	20	324
396	77	5.6	19	406
400	58	6.4	36	350
407	32	3.5	11	243

Number	MVD	%TVV	KI-67%	HGF/SF
410	173	13.9	33	620
417	46	5.8	14	588
425	157	12.5	32	995
433	53	4.5	8.8	473
436	91	4.8	15	185
437	91	9	2	465
442	123	7	15	475
446	84	4.3	19	330
451	58	3.5	5	129
455	47	3.4	11	120
462	36	5.3	6.8	84
474	111	4.9	5.6	132
475	?	?	19.5	1604
477	80	7.2	5	396
478	72	8.1	29	677
489	96	6.2	13.5	78
494	88	4.1	?	319
503	229	10.5	6.5	621
505	85	3.9	34	530
511	49	4.5	25	393
512	148	6.4	22	513
515	45	4.8	11	456
516	28	3	25	383
517	60	5.1	13	345
522	142	9.1	16	798
529	82	5.1	38	427
531	122	10.9	20	462
533	87	11.7	50	915
547	60	5.7	42	135
560	156	10.6	24	310
561	60	6.3	20	60
565	166	6.6	36	279
578	82	6.7	17	297

Key

HGF/SF ng/100mg total protein.