Hypoxia and Angiogenesis in Colorectal Liver Metastases

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By

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ABSTRACT

HYPOXIA AND ANGIOGENESIS IN COLORECTAL LIVER METASTASES
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Introduction

Despite surgical resection being the only potential cure for colorectal liver metastases, not all patients are suitable for operative intervention. Most patients who undergo surgery die of recurrent disease. Understanding tumour behaviour may identify patients who might benefit from targeted adjuvant therapy.

Tumour hypoxia and angiogenesis are a negative prognostic factor in various solid tumours. Carbonic anhydrase IX (CAIX), epidermal growth factor receptor (EGFR), matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9) have been associated with poor outcome. The aim of this study was to determine the prognostic significance of CAIX, EGFR, MMP-2 and MMP-9 in patients with colorectal liver metastases.

Methods

Patients having undergone a liver resection for colorectal liver metastases at our institutions between 1993 and 1999 were included. Standard immunohistochemical techniques were used to study the expression of CAIX, tumour necrosis, EGFR, MMP-2 and MMP-9 in 5μm tumour sections from paraffin-embedded tissue blocks. These factors were correlated with clinico-pathological data, microvessel density and vascular endothelial growth factor expression using the chi-squared test. The Kaplan-Meier method and log rank test were used for univariate survival analysis and the Cox proportional hazard model for multivariate survival analysis.

Results

181 patients, male n=121, female n=60, aged between 25-81 years (mean 61 years) were included. Tumour necrosis, membranous CAIX and membranous EGFR were significantly correlated with poor survival on univariate (p=0.0002, p=0.002 and p=0.01 respectively) and multivariate analysis (p=0.019, p<0.0001 and p=0.027 respectively). MMP-9 significantly correlated with tumour edge contiguous vessels and central tumour vessels. MMP-2 correlated with membranous CAIX.

Conclusion

CAIX, EGFR and tumour necrosis are independent negative prognostic factor in patients with colorectal liver metastases. MMP-2 and MMP-9 may have an important role in tumour invasion.
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# CONTENTS

<table>
<thead>
<tr>
<th>Title</th>
<th>Page Numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corrected Title</td>
<td>i</td>
</tr>
<tr>
<td>Abstract</td>
<td>ii</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>iii</td>
</tr>
<tr>
<td>List of Contents</td>
<td>iv</td>
</tr>
</tbody>
</table>

## Introduction

<table>
<thead>
<tr>
<th>Title</th>
<th>Page Numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colorectal Cancer</td>
<td>3</td>
</tr>
<tr>
<td>Hypoxia</td>
<td>11</td>
</tr>
<tr>
<td>Hypoxia and Angiogenesis</td>
<td>14</td>
</tr>
<tr>
<td>Carbonic anhydrase IX</td>
<td>26</td>
</tr>
<tr>
<td>Epidermal Growth Factor Receptor</td>
<td>37</td>
</tr>
<tr>
<td>The Metalloproteinases</td>
<td>52</td>
</tr>
<tr>
<td>Hypoxia Inducible Factor-1</td>
<td>63</td>
</tr>
<tr>
<td>Hypoxia and Angiogenesis as targets for therapeutic strategies</td>
<td>69</td>
</tr>
<tr>
<td>Introduction to experimental techniques: immunohistochemistry</td>
<td>74</td>
</tr>
<tr>
<td>Summary of Experimental Data in Liver Metastases</td>
<td>78</td>
</tr>
<tr>
<td>Aims of the study</td>
<td>79</td>
</tr>
</tbody>
</table>

## Methods

<table>
<thead>
<tr>
<th>Title</th>
<th>Page Numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients and Ethics</td>
<td>80</td>
</tr>
<tr>
<td>Immunohistochemical Detection Kits</td>
<td>87</td>
</tr>
<tr>
<td>Materials and Methods: The Experimental Techniques</td>
<td>90</td>
</tr>
<tr>
<td>Methods for CAIX</td>
<td>95</td>
</tr>
<tr>
<td>Methods for EGFR</td>
<td>98</td>
</tr>
<tr>
<td>Methods for Metalloproteinase-2</td>
<td>104</td>
</tr>
<tr>
<td>Methods for Metalloproteinase-9</td>
<td>107</td>
</tr>
<tr>
<td>Methods for HIF-1 alpha</td>
<td>109</td>
</tr>
<tr>
<td>Standard Operating Procedures</td>
<td>114</td>
</tr>
<tr>
<td>Summary of Past Techniques used in this Series</td>
<td>118</td>
</tr>
</tbody>
</table>
List of Tables

Chapter 1
Table 1.1 Epidermal growth factor receptor-specific ligand binding 39
Table 1.2 EGFR mutations in solid tumours 45
Table 1.3 Classification of the Metalloproteinases 53

Chapter 2
Table 2.1 Summary of immunohistochemical visualisation methods 92
Table 2.2 Reagents contained in the immunohistochemistry detection systems 93
Table 2.3 Summary of primary antibodies for immunohistochemistry 94
Table 2.4 Summary of general reagents 94
Table 2.5 Substrate-chromogen preparation using the ChemMate system 100
Table 2.6 Streptavidin-biotin-peroxidase complex preparation 111
Table 2.7 Substrate-chromogen solution preparation 112
Table 2.8 Immunohistochemistry for VEGF 119

Chapter 3
Table 3.1 Patient survival data 122
Table 3.2 Clinicopathological factors and their prognostic value 122

Chapter 4
Table 4.1 Expression of CAIX 129
Table 4.2 Inter-observer variability 135
Table 4.3 CAIX expression and clinicopathological variables 136
Table 4.4 CAIX and MVD 136

Chapter 5
Table 5.1 Clinico-pathological variables and EGFR 146
Table 5.2 EGFR and MVD 147
Table 5.3 EGFR, CAIX and VEGF 147
Chapter 6

Table 6.1 Clinico-pathological variables and MMP-9 159
Table 6.2 MMP-9 and MVD 160
Table 6.3 MMP-9, CAIX, EGFR and VEGF 160
Table 6.4 Clinico-pathological variables and MMP-2 164
Table 6.5 MMP-2 and MVD 167
Table 6.6 MMP-2 and mediators of angiogenesis 167

List of Figures

Chapter 1
Figure 1.1 Structure of EGFR 38
Figure 1.2 EGFR and cell signalling pathways 42

Chapter 4
Figure 4.1 Membranous and cytoplasmic CAIX expression in a liver metastasis 130
Figure 4.2 CAIX immunoreactivity in NSCLC 130
Figure 4.3 Negative CAIX in a colorectal liver metastasis 131
Figure 4.4 Tumour edge and normal liver tissue 132
Figure 4.5 CAIX immunoreactivity in a bile duct 132
Figure 4.6 CAIX immunoreactivity in a gallbladder specimen 133
Figure 4.7 Necrosis in liver metastases at x4 magnification 134
Figure 4.8 Median membranous CAIX expression and survival 137
Figure 4.9 Median liver cytoplasmic CAIX expression and patient survival 137
Figure 4.10 Tumour necrosis and patient survival 138

Chapter 5
Figure 5.1 EGFR immunoreactivity in a colorectal liver metastasis 144
Figure 5.2 EGFR immunoreactivity in NSCLC 145
Figure 5.3 Negative EGFR immunoreactivity in NSCLC 145
Figure 5.4 Present versus absent membranous EGFR and survival 149
Figure 5.5 Present versus absent cytoplasmic EGFR and patient survival 149
Chapter 6

Figure 6.1 Tumour cell MMP-9 immunoreactivity
Figure 6.2 Negative MMP-9 immunoeexpression
Figure 6.3 Negative MMP-9 immunoreactivity in the stroma
Figure 6.4 Stroma + MMP-9 immunoreactivity
Figure 6.5 Stroma ++ MMP-9 immunoreactivity
Figure 6.6 Stroma +++ MMP-9 immunoreactivity
Figure 6.7 Cytoplasmic MMP-9 and patient survival
Figure 6.8 Stromal MMP-9 and patient survival
Figure 6.9 Tumour cell MMP-2 immunoreactivity
Figure 6.10 Stroma + MMP-2 immunoreactivity
Figure 6.11 Stroma ++ MMP-2 immunoreactivity
Figure 6.12 Stroma +++ MMP-2 immunoreactivity
Figure 6.13 Tumour cell MMP-2 and patient survival
Figure 6.14 Stromal MMP-2 and patient survival

List of abbreviations in alphabetical order

APC Adenomatous polyposis coli tumour suppressor gene
ARNT Aryl receptor nuclear translocator gene
ATP Adenosine triphosphate
bFGF b-fibroblast growth factor
CAIX Carbonic anhydrase IX
CEA Carcinogen Embryonic Agent
DAB 3, 3’-diaminobenzidine tetrahydrochloride
DCC Deletion Colon Cancer
DCIS Ductal carcinoma in-situ of the breast
ECM Extracellular matrix
EGF Epidermal growth factor
EGFR Endothelial growth factor receptor
Egr-1 Early growth response-1
ELISA Enzyme-linked immuno absorbent assay
Erks Extracellular regulated kinases
FA Folinic acid
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>5FU</td>
<td>5 fluorouracil</td>
</tr>
<tr>
<td>HIF-1α</td>
<td>Hypoxia-inducible factor-1α</td>
</tr>
<tr>
<td>HNPCC</td>
<td>Hereditary non polyposis colorectal cancer</td>
</tr>
<tr>
<td>HNSCC</td>
<td>Head and neck squamous cell carcinoma</td>
</tr>
<tr>
<td>HRE</td>
<td>Hypoxic response element</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus kinase</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MMP-2</td>
<td>Matrix metalloproteinase-2</td>
</tr>
<tr>
<td>MMP-9</td>
<td>Matrix metalloproteinase-9</td>
</tr>
<tr>
<td>MVD</td>
<td>Microvessel density</td>
</tr>
<tr>
<td>MMR</td>
<td>(DNA) mismatch repair</td>
</tr>
<tr>
<td>NGR</td>
<td>Neuregulins</td>
</tr>
<tr>
<td>NSCLC</td>
<td>Non-small cell lung cancer</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3 kinase</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PTB</td>
<td>Phosphotyrosine-binding (domains)</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>STATS</td>
<td>Signal Transducers and Activators of Transcription</td>
</tr>
<tr>
<td>TAF</td>
<td>Tumor angiogenic factor</td>
</tr>
<tr>
<td>TIMP</td>
<td>Tissue inhibitors of metalloproteinases</td>
</tr>
<tr>
<td>TGF-α</td>
<td>Transforming growth factor-α</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VHL</td>
<td>von Hippel Lindau</td>
</tr>
</tbody>
</table>
INTRODUCTION
Cellular proliferation is characterised by mitotic divisions alternating with periods of non-division. This cycle is strictly controlled at specific transition periods, by tumour suppressor genes that repress cell division and by proto-oncogenes that promote cell division. Uncontrolled cellular proliferation resulting from genetic mutations and disordered signal transduction pathways in response to carcinogens, results in the development of malignant tumours (Hanahan and Weinberg, 2000; Semenza, 2000a; Semenza, 2000b) (Cummings, 1994a; Cummings, 1994b).

The biological characteristics of malignant tumours due to altered gene expression and altered signal transduction are the focal point of much present-day research. One important characteristic of tumours is their ability to maintain nutrient supply by establishing their own vasculature. However, in the absence of adequate oxygenation or hypoxia, tumours are able to implement complex, adaptive survival strategies that are becoming increasingly associated with more aggressive phenotypes. Little is known about the biological characteristics of these tumours, which if more clearly understood could lead to the development of tumour-specific adjuvant therapy, especially for those patients unsuitable for surgery or at high risk of developing recurrent disease.

The following introductory pages outline the role of hypoxia, angiogenesis and extracellular remodelling in malignant tumours with particular emphasis on colorectal cancer and colorectal liver metastases, the tumour model studied herein. An introduction to the growth factors studied, including an overview of the methods used, the aims, and the objectives of the study are presented. The methods’ chapter details the study population and generic techniques utilised in the study. The results for each
growth factor are presented as individual chapters. A working hypothesis, discussion and future work are presented in the concluding chapter.

1. COLORECTAL CANCER

1.1 Epidemiology

There are 20,000 colorectal cancer deaths per annum, making it the third commonest cancer the UK, after lung and breast cancer (Scholefield, 2000). Worldwide, approximately 10 million new cases are diagnosed per year, with an incidence of 9.4% of all cancers in men and 10.1% in women, resulting in approximately 394,000 deaths per year (Boyle and Langham, 2000). Male to female ratio of incidence appears to be approximately equal, with peak age incidence occurring between 60-70 years (Boyle and Langham, 2000).

The incidence of colorectal cancer is greater in western societies and has, by population studies, been associated with environmental factors, including socio-economic and cultural practices, such as diet - consumption of animal fat and low fibre intake (Willett et al., 1990)- and low physical activity (Giovannucci et al., 1996).

1.2 Genetics and Pathology

The adenoma-carcinoma cycle is the genetic model for the development of colorectal cancer (Reviewed in (Ilyas et al., 1999; Boland et al., 2000; Cunningham and Dunlop, 1994; Hardy et al., 2000)).
Two separate pathways are believed to occur in the carcinogenic process. In the first mechanism postulated, sporadic mutations to the adenomatous polyposis coli tumour suppressor gene APC (5q) lead to sequential chromosomal instability of genes required for regulation of cell growth and transduction pathways, such as the k-ras oncogene (12p), Deletion Colon Cancer DCC (18q) and p53 (17p) which lead to the progression of an adenoma into a carcinoma. Somatic mutations to APC account for approximately 80% of all sporadic colorectal cancers; germline mutations to ACP which require only one further “hit” or inactivating event (as opposed to two in a normal gene) to promote the development of adenomas, result in a condition known as familial adenomatous polyposis coli.

The second mechanism involves mutations to the DNA mismatch repair mechanism (MMR). This system normally targets DNA with base pairing errors for repair, which is particularly important in genes involved in cell growth regulation. Mutations to the MMR proteins results in hypermutable DNA repeat sequences (known as microsatellite instability), mutations that can lead to loss of tumour suppressor gene function, gain of oncogene function, and eventually autonomous cell growth. Somatic mutations to MMR proteins account for approximately 15% of all colorectal cancers; germline mutations to MMR proteins can lead to hereditary nonpolyposis colorectal cancer (HNPCC), which accounts for approximately 3% of colorectal cancers.

Colorectal cancers are classified according to the TNM or modified Dukes’ classification system (Hardy et al., 2000).
1.3 Presentation and management

The main symptoms of colorectal cancer are rectal bleeding, altered bowel habit, palpable rectal or right-sided abdominal mass associated with weight loss and iron deficiency anaemia (Hobbs, 2000). Investigation of symptoms, by sigmoidoscopy / colonoscopy or radiological imaging (barium enema, CT colography), is followed by surgical and/or oncological care within a multidisciplinary team framework.

The liver is the most common organ for secondary deposits deriving from colorectal primary tumours, due to haematogenous spread from the gastrointestinal tract to the liver via the portal vein. Up to 50% of all patients with colorectal cancer develop liver metastases. Liver metastases only may account for deaths in as many as 38% of these patients (Fong et al., 1997). Surgical resection of liver and lung secondaries can result in cure (Fong et al., 1997), but only 20-25% of patients with liver involvement are suitable for hepatic resection (Geoghegan and Scheele, 1999).

1.3.1 Surgical resection

Surgical resection of hepatic metastases from colorectal origin is to date, the only potentially curative mode of treatment. One third of patients survive 5 years upon complete resection of liver lesions (Scheele et al., 1995; Fong et al., 1999; Nordlinger et al., 1996; Taylor et al., 1997). 23% may survive 10 years and 18% 20 years. Disease free survival is approximately 15%-20% after 5 years (Nordlinger et al., 1996; Taylor et al., 1997). This compares very favourably to untreated patients whose median survival is 6-12 months and to those treated with chemotherapy whose survival is 12-18 months. The main risk of postoperative complications of hepatic
resections appears to be related to the extent of liver resected, (Doci et al., 1995) and mortality is in the region of 2-5% (Fong et al., 1999) (Nordlinger et al., 1996; Doci et al., 1995).

Prognostic factors to predict outcome after hepatic resection for colorectal metastases have been documented by various series and used to devise scoring systems of clinical risk.

Lymph node invasion and tumour resection margin of <10mm have consistently been associated on multivariate analysis with poor outcome in the three largest liver resection series: Jaeck et al encompassing 1818 patients in a multicentre study, (Jaeck et al., 1997) Nordlinger in a multicentre experience of 1568 patients in 85 institutions in France (Nordlinger et al., 1996) and Fong et al in their analysis of 1001 consecutive hepatic resections (Fong et al., 1999), and in some smaller studies (Shirabe et al., 1997).

Fong et al, in addition to node status of the colorectal primary and operative tumour clearance (5 year survival of 20% associated with positive margins) identified <12 month interval between primary and metastatic disease, >1 number of tumours, >200ng/ml preoperative Carcinogen Embryonic Agent (CEA), >5cm diameter for the largest tumour as important prognostic factors, and allocated a one point scoring system for each parameter: 60% of patients with 0 points survived 5 years; no patients survived 5 years with a score of 5. Multiple and bilobar metastases were associated with a 23% and 29% 5-year survival respectively.
Except for disease distribution, these parameters were also identified as prognostic of survival on multivariate analysis by Nordlinger (Nordlinger et al., 1996) who proposed another scoring system to stratify patients into three groups of expected survival and cure: low risk, (79% 2-year survival), intermediate risk (60% 2-year survival) and high risk (43% 2-year survival).

1.3.2 Chemotherapy

Chemotherapy can either be of curative intent, when given as post-operative adjuvant therapy to eradicate micrometastases, or palliative, for symptom control in cases of advanced disease. The thymidylate synthase inhibitor, fluorouracil, is the principal cytotoxic agent used, which inhibits thymidine and hence DNA/RNA synthesis. It is often used in combination with folinic acid due to their synergistic effects, with observed benefit in the treatment of Dukes’ C cancers, although not as clear for Dukes’ B cancers (Midgley and Kerr, 2000) (Young and Rea, 2000). Other cytotoxic agents are being evaluated, but the very nature of their cytotoxicity means that they do not exclusively target cancer cells and patients are therefore more likely to develop side effects.

Novel treatment strategies include immunotherapy, which aims to generate cell-mediated immune response against tumour cells, and gene therapy to either correct single gene mutations such as p53 or deliver enzyme pro-drugs into tumours cells to locally activate metabolites. Specific anti-cytokine agents are the latest treatment strategies being developed, working towards the aim of specific, targeted therapies (Chung-Faye and Kerr, 2000).
Regimes used for patients with colorectal cancer have been reviewed by the Third International Conference: Perspectives in Colorectal Cancer held in Dublin 2001 and based on large trial evidence have recommended treatment strategies as outlined below (Van Cutsem et al., 2002).

Dukes’ C colorectal cancers should be treated with combination therapies of 5-flourouracil and folinic acid or levimasole infusion for 6 – 8 months, since trials have shown to reduce the relative risk of mortality from 22%-39% (Van Cutsem et al., 2002). Three drug combinations were not shown to confer any further benefit, and in view of increased side effects, not recommended. The Roswell Park Regime and the Mayo Clinic regime are the standard treatment administration schedules in the USA with the Mayo Clinic regime being the standard in Europe. The former comprises 6 weeks’ treatment followed by two weeks’ rest over four cycles, and the latter involved drug administration for days 1-5 for 4-5 weeks over a six- month period (Van Cutsem et al., 2002).

Topoisomerase I inhibitors- irinotecan (prevents cell division) and platinum compounds - oxaliplatin (third generation platinum compound with a 1,2-diaminocyclohexane carrier ligand, which like other platinum compounds acts mainly by causing inter and intra- strand crosslinks in DNA thus preventing DNA replication) (Wiseman et al., 1999) - and fluoropyrimidines- capecitabine and uracil/tegafur - are current regimes. Intravenous irinotecan is licensed for first line chemotherapy in combination with 5 (FU)/FA or as second line monotherapy, and has been shown to potentiate the effects of 5FU/FA resulting in better tumour response and better quality of life, which were also documented with its use as a second line agent. Oxaliplatin
also potentiates 5FU/FA and improves response rates, thus it is also licensed for
combination first line therapy with 5FU/FA. Capecitabine has been shown to be as
effective as 5FU/FA infusions without the side effects of intravenous drug
administration. No clear data are available regarding improved survival for these
agents. The National Institute for Clinical Excellence guidelines (National Institute
for Clinical Excellence, March 2002) however, have controversially not
recommended first line therapy with irinotecan or oxaliplatin unless as part of clinical
trials. Instead, the use of irinotecan as second line monotherapy, and combined
5FU/FA with oxaliplatin in patients with potentially resectable liver metastases (as
tumour bulk has been shown to decrease) has been recommended (Anonymous –
Drugs and Therapeutics Bulletin- 2002).

Treating all patients with stage II (Dukes’ B) colorectal cancer with adjuvant therapy
has not reached general consensus, and was not recommended at the Dublin
Conference due to lack of evidence as to its effectiveness. High-risk patients may
benefit, but the problem lies in identifying patients at high risk; biomolecular markers
will potentially enable such patients to be identified and treated with targeted therapy
in the future (Van Cutsem et al., 2002).

Treatment for patients post liver resection for liver metastases has not been
recommended for routine use but may be of benefit in some cases. Similarly, post-
operative intra-portal chemotherapy and immunotherapy currently lack sufficient
evidence to be recommended as standard practice, but certain select patients may
benefit (Van Cutsem et al., 2002).
Neoadjuvant chemotherapy, using combinations of 5FU, leucovorin, oxaliplatin and irinotecan may facilitate surgical intervention in colorectal liver metastases initially classified as unresectable, with considerable survival advantages for patients. Adam et al reported increased tumour resectability and improved patient survival upon downstaging liver tumour bulk using combination chemotherapy -5FU and leucovorin in combination with oxaliplatin (70%), irinotecan (7%) or both (4%)- (Adam et al., 2004a) (Adam et al., 2004b). In the first published study, Adam described secondary hepatic resection in 138 patients from 1104 initially unresectable tumours downsized by neoadjuvant therapy. Five-year survival amongst these patients was reported as 35-40%, in stark contrast from the median survival of 20 months associated with chemotherapy alone (Adam et al., 2004a). In the second study, Adam et al reported increased survival rates post liver resection if disease had been controlled or showed good response to neoadjuvant therapy, in contrast to patients in whom disease progression had occurred prior to surgery (Adam et al., 2004b). They surmise that liver resection is thus feasible if disease is downstaged or controlled by chemotherapy, but patients in whom disease progression is evident despite chemotherapy, the outcome is poor despite surgical intervention.

The management of rectal cancer principally relies on good surgical technique. Total mesorectal excision is the gold standard for mid to low-lying tumours and all tumours need circumferential excision. Adjuvant or neoadjuvant treatment in the form of preoperative radiotherapy, preoperative radiotherapy plus chemotherapy or postoperative radio and chemotherapy, have been recommended (Van Cutsem et al., 2002).
Despite surgical resection being the only potentially curative treatment for colorectal liver metastases, not all patients are suitable for operative intervention. Of those patients who do undergo surgery, most will die of recurrence disease; hence the need to elucidate the biological characteristics of these tumours and develop targeted adjuvant therapy. Tumour hypoxia and angiogenesis are negative prognostic factors in various solid tumours.

2. HYPOXIA

2.1 General principles

The ability of a tumour to successfully grow and metastasise is defined by its ability to establish and sustain its own blood supply (angiogenesis), invade the surrounding tissue, intravasate into the local circulation and subsequently seed to secondary target organs. These cellular processes are facilitated by the breakdown and remodelling of the extracellular matrix through certain key enzymes, influenced by the physiological characteristics of the surrounding microenvironment and promoted by hypoxia.

Small tumours (<1cm) are well perfused, but rapid proliferation results in tumours outgrowing their blood supply leading to areas of chronic hypoxia, which in-turn, stimulate various adaptive mechanisms enabling tumours to survive (Folkman, 1995). Hypoxia up-regulates genes which promote neovascularisation, genes which promote changes in intracellular cell metabolism and genes to enable whole organism adaptation to hypoxic conditions by processes such as vasodilatation and erythropoiesis. Gene up-regulation is often facilitated by the activation of
transcriptional factors such as hypoxia-inducible factor-1α (HIF-1α) (Blancher and Harris, 1998; Shih and Claffey, 1998) (Richard et al., 1999) (Semenza et al., 1997). One particular molecule which is up-regulated by hypoxia is carbonic anhydrase IX (CAIX), an enzyme involved in the acidification of tumour extracellular matrix and cellular adhesions. It has thus been described as a marker of hypoxia and correlated with poor patient survival in a variety of solid tumours (Beasley et al., 2001; Bui et al., 2003; Chia et al., 2001; Giatromanolaki et al., 2001; Hui et al., 2002; Loncaster et al., 2001; Maseide et al., 2004; Swinson et al., 2003).

Hypoxia has been shown to activate programmed cell death, or apoptosis in vitro (Rat1 fibroblasts cultured in anaerobic conditions sustained marked cell loss as compared with cells cultured in aerobic conditions (Graeber et al., 1996)), by up-regulating anti-apoptotic genes such as bcl-2 (Bonn, 2000) and by naturally selecting cells either deficient in p53-mediated apoptotic pathways, or displaying mutated forms of the p53 gene. Graeber et al, using mouse embryonic fibroblasts demonstrated that those expressing p53 activated by oncogenes were very susceptible to apoptosis within a hypoxic environment comparable to that found in solid tumours, whereas those cells without p53 remained largely viable (Graeber et al., 1996). In vivo work has demonstrated that areas of high apoptotic activity in tumours correspond to regions of hypoxia in tumours with wild-type p53, whereas tumours without p53 did not demonstrate areas of apoptosis associated with hypoxia (Graeber et al., 1996). These findings may also explain why some hypoxic tumours are resistant to radio- and chemotherapy (Graeber et al., 1996; Marti et al., 2000; Semenza, 2000) (Birner et al., 2001) (Kondo et al., 2001) (Kress et al., 1998).
2.2 Necrosis

If tumours are unable to maintain an adequate blood supply (Bussolati et al., 2000; Rofstad and Halsor, 2002), coagulative necrosis can develop. Necrosis encompasses changes to the morphology of a cell after its death, whereby the original shell-like structure is preserved but there is no metabolic activity. Loss of barrier function allows inflammatory cell infiltration which, in addition to the release of intracellular enzymes secondary to cellular devitalisation, results in cellular liquefaction (Govan et al., 1991; Groepel, 1992; Leek et al., 1999). This process may either occur as a result of chronic hypoxia and ischaemia secondary to excessive tumour growth or due to acute cellular hypoxia secondary to the sudden occlusion of immature neovessels (Hemmerlein et al., 2001). However, the converse may also be true, such that the development of necrosis may be associated with more aggressive, rapidly growing tumours, as angiogenic induction cannot maintain adequate vascularisation. These highly necrotic tumours may be associated with areas of high angiogenic activity (elevated microvessel density counts, MVD) and have indeed been correlated with poor prognosis in breast cancer (Leek et al., 1999).

2.3 The measurement of tumour hypoxia and necrosis

Tumour hypoxia has been measured in animal models and in human tumours by two principle methods. Direct measurements of tumour oxygenation can be obtained using Eppendorf polarographic measurements of the partial pressures of oxygen ($pO_2$). Values $< 5$ mm Hg have been defined as representative of tissue hypoxia (Milross et al., 1997). Some authors argue that measurements undertaken in areas of necrosis
should be excluded, as they do not include viable hypoxic clonal cells responsible for
the growth and behaviour of the tumour (Fenton et al., 2002; Milross et al., 1997).

Indirect measurements of tissue hypoxia and its spatial distribution within solid
tumours involve the immunohistochemical detection of endogenous markers of
hypoxia, such as nitrimidazole compounds (EF5, pimonidazole) (Evans et al., 2001;
Fenton et al., 2002), which bind to hypoxic cells, and the more recently described
carbonic anhydrase IX (Pastorek et al., 1994), which is up-regulated by hypoxia. The
expression of these surrogate markers of hypoxia has been correlated with the
expression of cytokines representative of other biological processes (for instance,
microvessel density for angiogenesis) in order to establish the behaviour of a
particular tumour at a certain point in time (Evans et al., 2001).

3. HYPOXIA AND ANGIOGENESIS

Angiogenesis is the formation of new blood vessels from a pre-existing vasculature,
to maintain adequate oxygenation and nutritional support for tumours to grow beyond
2-3mm diameter (Folkman, 1971). Angiogenesis also occurs as a normal
physiological response in wound healing, bone formation, in the placenta, during
embryogenesis, and is seen in non- malignant conditions such as psoriasis,
rheumatoid arthritis, diabetic retinopathy, haemangiomas and atherosclerotic plaque
formation (Bouck et al., 1996). Angiogenesis has been associated with tumour cell
proliferation and has been shown to be a useful prognostic marker, often of poor
outcome in various tumours, including breast, prostate, ovary (Ellis and Fidler, 1996),
non- small cell lung cancer (Cox et al., 2000), malignant mesothelioma (Edwards et

The concept of angiogenesis is not new, since early animal models clearly describe the importance of neovascularisation for tumours to progress from a slow, avascular phase to a rapid growth phase (Bouck et al., 1996) (Knighton et al., 1976; Conman and Sheldon, 1946). Notable examples include the implantation of ocular tumours in the anterior chamber of the guinea pig eye with resulting endothelial proliferation of the vascular iris (Gimbrone JR et al., 1973), and the implantation of Brown-Pearce epithelioma in the rabbit cornea with resulting corneal vascularisation (Gimbrone JR et al., 1974). The presence of a pre-existing vasculature was imperative, since tumours implanted away from endothelia failed to grow and remained “dormant” (Gimbrone JR et al., 1972) (Williams, 1951).

However, it was not until the work of J. Folkman in the 1960s and 1970s that a true understanding of angiogenesis was gained. The works of Folkman and colleagues concluded that solid tumours relied on angiogenesis for growth beyond 2mm in size, demonstrated by impaired thyroid tumour growth in culture (Folkman et al., 1966) in response to certain, diffusible agents (Folkman, 1971). The first such agent was described as “Tumor Angiogenic Factor”(TAF) (Folkman, 1971), currently known as vascular endothelial growth factor. Since then, numerous angiogenesis-promoting factors have been described. By the 1990s it was clear that angiogenesis was modulated by a balance between pro and anti-angiogenic cytokines (Folkman, 1995).
Present day research concentrates on the expression of a variety of cell surface molecules and important signalling pathways, which activate cell proliferation and locally released factors involved in basement membrane lysis.

3.1 Angiogenesis and the remodelling of the extracellular matrix

3.1.1 Establishing a vascular network

During the pre-vascular, growth-restricted stage of a tumour, endothelial cells are quiescent, arguably due to a local equilibrium between pro and anti-angiogenic factors (Folkman, 1995). Endothelial proliferation is stimulated when tumour cells switch to an angiogenic phenotype, which is believed to result from a change in equilibrium between angiogenesis modulators in favour of pro-angiogenic factors, which leads to mitogenic stimulation of the microvasculature.

Upon stimulation, endothelial cells from normal host tissue migrate towards the angiogenic source, due to a combination of direct cell-to-cell interactions with growth promoting cytokines, and in response to cytokine-driven release of chemotactic factors from the endothelium, macrophages, intracellular stores or the extracellular matrix. Growth is initially promoted by good perfusion gradients allowing for nutrient exchange, and a paracrine autoloop is generated by the activated endothelium *per se* to encourage further growth (Folkman and Klagsbrun, 1987; Folkman, 1995; Folkman, 1995).

The developing capillary sprout is hyper-permeable, particularly due to over-expression of vascular endothelial growth factor (VEGF) (Bhujwalla et al., 2001).
This leads to the extravasation of plasma proteins, most notably fibrin, into the extracellular matrix creating a fibrin network in the tumour stroma to attract and sustain the growth of endothelial cells, thus establishing a new vascular bed (Dvorak et al., 1995) (Ferrara, 1996; Senger et al., 1993). This fibrin skeleton however also restricts cell movement, thus for subsequent vascular invasion, migration and differentiation to occur sufficient fibrin needs to be degraded without compromising its structural support. Proteolytic enzymes (notably the matrix metalloproteinases and plasminogens) and cell adhesion molecules within the extracellular matrix (integrins, selectin) are mobilised (Gasparini, 1996) that respectively digest the extracellular matrix. This enables vessels to proliferate and further growth factors to be released (Folkman and Klagsbrun, 1987; Toi et al., 1996), facilitating endothelial cell-to-extracellular matrix-interactions, particularly with collagen and fibronectin, to further sustain the firm cytoskeleton for the growing vascular bed (Senger et al., 1993). The developing vessels are morphologically different from normal vessels. Complex growth patterns develop, such as arterio-venous shunts and blind-ending vessels, possibly in a tumour-specific manner (Konerding et al., 2001; Folkman and Klagsbrun, 1987).

3.1.2 The extracellular matrix - degradation and microenvironment

It is well recognised that cellular processes involved in tumour growth are determined by the surrounding microenvironment. One very important factor is the cellular pH.

The extracellular pH of tumours tends to be more acidic than normal tissue (pH 7.0 compared with 7.5), which is essential for the optimal functioning of many ECM-
modulating factors, especially the matrix metalloproteinases such as MMP-2, and MMP-9, which have been shown to be important for tumour cell invasion and metastasis in colorectal cancer (Waas et al., 2002; Waas et al., 2003). Hypoxia-induced changes in cell metabolism, and changes in \( \text{Na}^+/\text{H}^+ \) ion exchange mechanisms (Reshkin et al., 2000; Webb et al., 1999) exert an important role in the generation of an acidic extracellular environment, as detailed below.

Under hypoxic conditions, normal oxidative phosphorylation cannot take place as oxygen is not available as the final electron carrier in the respiratory chain, therefore cellular metabolism must change to glycolysis to produce energy (ATP). This increase in glycolytic metabolism was first described by Warburg in 1930, and therefore called the Warburg effect (Semenza, 2000). Since oxidation of electron carriers cannot take place, these carriers become reduced, generating reactive oxygen intermediates that may activate transcription factors such as hypoxia inducible factor-1 (HIF-1) (Shih and Claffey, 1998). Hypoxia promotes such glycolytic metabolism by up-regulating the transcription of glycolytic enzymes such as lactate dehydrogenase (Blancher et al., 2001) and glucose transporters such as GLUT-1 and GLUT-3. This provides ATP through the anaerobic pathway, generates lipid and nucleotide precursors and facilitates the entry of glucose into cells. The hypoxia pathway per se may also be involved in the generation of nucleotide precursors for ATP formation, since HEPA-1 mouse hepatoma cell lines deficient in components of this pathway, notably HIF-1β, had lower ATP levels than hepatomas with wild type HIF-1β (Griffiths et al., 2002). It has been suggested that cells resistant to hypoxia through differences in mitochondrial function decrease their oxygen consumption, maintain stable ATP
levels and stable electrical activity across the mitochondrial membrane in contrast to hypoxia-sensitive cells (Turcotte et al., 2002).

Through glycolysis, glucose is converted into lactate, contributing towards an acidic extracellular environment. Indeed metastatic breast cancer cell lines have been shown to produce high lactate levels (Bhujwalla et al., 2001) and lactate has also been shown to correlate with metastatic tumours in cervical (Semenza, 1998) and head and neck cancer (Semenza, 2000). During hypoxia-induced glycolysis, protons are actively extruded from the cell, further contributing to extracellular acidification (Webb et al., 1999). Hypoxia also up-regulates carbonic anhydrase IX, which has an important role in pH maintenance through ion exchange mechanisms (see Carbonic anhydrase IX).

Further research into the role of ion exchange mechanisms in tumorigenesis has yielded interesting results. *In vitro* work using increasingly neoplastic human breast cancer cell lines demonstrated that under serum-starved conditions, the activity of the Na⁺/H⁺ ion exchanger was up-regulated in tumour cells, thus increasing their capacity to acidify the extracellular space. A reversal in the normal regulating mechanisms and greater affinity for protons by the exchanger were postulated as possible causes. The motility and invasiveness of these cells was also increased. In contrast, normal cells down-regulated the activity of the ion exchanger (Reshkin et al., 2000).

**3.2 Invasion and metastases**

It is widely accepted that tumour cells displaying angiogenic potential coupled with the capacity to proliferate and invade, by interacting with angiogenesis-modulating factors in a microenvironment that facilitates further cell growth, will characterise a
biologically successful tumour and determine metastatic potential (Folkman and Klagsbrun, 1987; Folkman, 1995) (Ellis and Fidler, 1996).

The development of metastases is a very complex, sequential cascade of events that enables a subpopulation of tumour cells to survive at a site distant to the primary lesion. Angiogenesis facilitates metastatic progression by enabling tumour cells to seed via highly permeable vessels which do not possess the structural complexities of the mature vascular system (Dvorak et al., 1995); higher density of these new vessels, or microvessel density, should in theory, therefore, correlate with increased metastatic potential and clinically, with poorer outcome, but as discussed later, this does not appear to be as straightforward.

An angiogenesis-dependent successful completion of the metastatic cascade is evidenced by lung metastases in mice remaining dormant in the absence of neovascularisation, and depends on:

1) The biological characteristics of each tumour; for example, in-situ hybridisation of pro-angiogenic genes in metastatic and non-metastatic tumours demonstrated higher expression of VEGF in metastatic tumours, especially in a sub-population of cells at the invasive tumour edge (Kitadai et al., 1995).

2) The ability of cells to survive at a distant site (Fidler et al., 1978; Poste and Fidler, 1979) (Ellis and Fidler, 1996).
3) The biological characteristics of the distant site (Ellis and Fidler, 1996; Folkman, 1995) (Gasparini, 1996). For instance, implantation of tumour cells in different sites demonstrated different patterns of expression of pro-angiogenic factors in relation to the site of implantation (gastric cancer cells implanted in gastric mucosa showed greater VEGF expression than cells implanted in ectopic tissue)(Singh et al., 1994; Takahashi et al., 1996).

4) The inter-relationship between the primary tumour, angiogenesis and the development of metastases.

The latter has been reviewed by Folkman (Folkman, 1996) and Zetter (Zetter, 1998) but in summary, the temporal presentation of metastases in relation to the primary tumour, have been believed to be related to modulation of angiogenesis. Dormancy was attributed to a balance between cellular proliferation and apoptosis when angiogenesis was inhibited (re-affirmed by the discovery of angiostatin, an anti-angiogenic agent). The clinical presentation of metastases may be related to the effects of the primary tumour on the expression of angiogenesis-modulating factors. For example, the primary tumour may suppress the development of the metastasis in the case of dormant colonies; failure to suppress may cause concomitant primary and metastatic disease or presentation with metastases and an occult primary; removal of the primary may cause regression of metastases (if it relied on growth factors released from the primary). Alternatively, the secretion of anti-angiogenic factors by metastases could influence the primary tumour.
3.3 The role of hypoxia in angiogenesis

Various experimental findings provide evidence for the role of hypoxia in promoting angiogenesis:

1) Increased levels of pro-angiogenic VEGF mRNA in hypoxic cells (Rofstad and Danielsen, 1998).

2) Increased expression of VEGF in perinecrotic tumour regions (Rofstad and Danielsen, 1998) and in regions of ischaemia (Marti et al., 2000).

3) The up-regulation of VEGF receptors located on endothelial cells (Marti et al., 2000).

4) Increased number of tumour-related capillaries under hypoxic conditions associated with increased rates of VEGF secretion (Rofstad and Danielsen, 1998).

5) Increased expression of HIF-1 protein as demonstrated by immunohistochemistry in a variety of solid tumours and not in the corresponding normal tissue by different research groups, including cancers of the prostate, lung, colon, breast and cervix; in early malignancy such as breast cancer in-situ (Zhong et al., 1999; Talks et al., 2000) and in bronchial metaplasia (Giatromanolaki et al., 2001).
4. HYPOXIA AND ANGIOGENESIS AS NEGATIVE PROGNOSTIC FACTORS - THE EVIDENCE

4.1 Hypoxia

Tumour cells with good adaptive mechanisms to hypoxia and resistance to apoptosis may characterise the most aggressive phenotypes through natural selection (Kondo et al., 2001). However, it may be that the most aggressive phenotypes promote poor tumour oxygenation and thus more hypoxic tumours.

Early investigation of hypoxia and survival was carried out in cervical cancer. Hockel et al. and Birner et al. in three separate studies correlated hypoxia and HIF-1 over-expression with poor prognosis: firstly cervical tumours with an intramural oxygenation of <10 mm Hg obtained by electrode probes correlated with increased local disease invasion and metastatic spread, whereas tumours with >10 mm Hg oxygenation correlated with higher 6-year survival rates (Hockel et al., 1996).

Secondly, HIF-1 over-expression in early stage invasive cervical cancer was found to be an independent prognostic marker, associated with decreased overall survival and disease-free survival (Birner et al., 2000). Thirdly, HIF-1 in combination with mutant p53 was found in epithelial ovarian tumours to be associated with a marked reduction in survival (mean 251 days as compared with 2129 days in patients with tumours not over-expressing HIF-1 and mutant p53) (Birner et al., 2001). More recently, Fyles et al. prospectively demonstrated using Eppendorf electrode measurements of cervical tumours, that hypoxia is an independent prognostic factor for poor progression-free...
survival in patients with node negative, large tumours of the cervix, and further proposed that such measurements should become routine clinical practice (Fyles et al., 2002).

Tumour hypoxia has also been identified as a negative prognostic marker in ovarian (Birner et al., 2001), and non-small cell lung cancer (Giatromanolaki et al., 2001) and has been associated with radio- and chemotherapy resistance in oesophageal cancer (Koukourakis et al., 2001). Radiotherapy requires oxygen for optimum efficacy, and various chemotherapeutic agents generate oxygen free radicals (Blancher and Harris, 1998). Koukourakis et al (Koukourakis et al., 2001) showed that incomplete response to photodynamic therapy and radiotherapy in oesophageal cancer correlated with high expression of HIF-1α and defective bcl-2, an apoptosis-inducing protein which, in abundance, has been associated with good survival in poorly vascularised tumours (Giatromanolaki et al., 2001). However, the relationship of these findings to overall survival was marginal. Response to chemotherapy in a study of epithelial tumours did not, however, correlate with over-expression of HIF-1α (Birner et al., 2001).

Tumour necrosis has also been associated with poor outcome in early stage non-small cell lung cancer (NSCLC) (Swinson et al., 2002) and in malignant mesothelioma (Edwards, et al, *in press*), but no difference was found in ductal carcinoma in-situ of the breast (DCIS) (Leek et al., 1999). Although a positive correlation between tumour size, increasing patient age and increasing tumour stage was found in breast DCIS, only early tumour stage was found to correlate in NSCLC (Swinson et al., 2002); there was no correlation with tumour stage in malignant mesothelioma, but the sample population was small (n=48) (Edwards, et al, *in press*).
4.2 Angiogenesis

Highly vascularised tumours are more likely to metastasise, by increasing vascular surface area and the ability of cells to reach the circulation (Folkman, 1995; Gasparini, 1996).

Historically, a variety of systems to grade the vascularity of tumours have been devised involving vascular densities (Brem et al., 1972), the use of endothelial markers (Srivastava et al., 1988), and counting microvessels in order to record the microvessel density (Weidner et al., 1991). High vessel counts have been repeatedly noted at the periphery of tumours and decrease towards the centre (Konerdig et al., 2001) and correlated with poor outcome (McCulloch et al., 1995).

Microvessel density has been shown by various studies to be an important prognostic indicator, often correlated with poor outcome in most solid tumours (Ellis and Fidler, 1996; Folkman, 1995; Gasparini, 1996; Bicknell and Harris, 1996; Ikeda et al., 1999; Edwards et al., 2001; Cox et al., 2000). Although extensive research into microvessel density and prognosis in colorectal cancer has been undertaken, an extensive review by Sutton et al (CD Sutton, 2004, unpublished data) demonstrated that there are many inconsistencies in the results, largely explained by methodological differences between papers.
5. CARBONIC ANHYDRASE IX

Carbonic anhydrases are transmembrane zinc metalloenzymes that catalyse the hydration of carbon dioxide into bicarbonate and a hydrogen ion.

\[ \text{CO}_2 + \text{H}_2\text{O} \leftrightarrow \text{HCO}_3^- + \text{H}^+ \]

Carbonic anhydrases (CA) participate in numerous physiological processes, including acid-base and electrolyte balance, respiration, and the formation of many secretory fluids throughout the body.

Fourteen isoenzymes have been characterised, each differing in tissue expression and distribution (Nishimori and Onishi, 2001). Intracellular isoforms include cytosolic CAI, CAII, CAIII, CAVII and mitochondrial membrane bound CAV (involved in gluco and ureaneogenesis) (Nishimori and Onishi, 2001). Extracellular isoforms comprise CAIV, secretory CAVI (found in saliva) and transmembrane CAIX, XII, XIV (Nishimori and Onishi, 2001). CAVIII, CAX and CAXI do not have catalytic properties, as they lack three histidine residues in the active site of the CA domain (Nishimori and Onishi, 2001; Nogradi, 1998; Lönnerholm et al., 1985; Supuran and Scozzafava, 2000).

Carbonic anhydrase IX was selected for evaluation in this study as it has been proposed as an endogenous marker of hypoxia, supported by the high correlation between the expression of CAIX and areas of hypoxia in cervical carcinoma (Loncaster et al., 2001), and its membranous, peri-necrotic distribution, such as in breast cancer (Chia et al., 2001), with comparable patterns of expression to
pimonidazole, an established marker of hypoxia. (Ivanov et al., 2001; Wykoff et al., 2001).

Carbonic anhydrase IX was originally described as a tumour associated gene product identified in HeLa cell lines from human carcinoma of the cervix (Opavský et al., 1996). The genomic sequence subsequently elucidated, by Opavsky et al 96 (Opavský et al., 1996).

5.1 STRUCTURE OF CAIX

Carbonic anhydrase IX is a transmembrane, acidic glycoprotein of 54/58 kD molecular weight and 459 amino acids length, consisting of an N terminal proteoglycan region (amino acids 1-37), an extracellular domain (amino acids 38-414) within which is located the carbonic anhydrase domain (amino acids 135-391), a transmembrane domain (amino acids 415-434), and a C-terminus (amino acids 435-459) consisting of a transmembrane cell surface anchor and an intracytoplasmic tail (Opavský et al., 1996; Pastorek et al., 1994).

The CAIX gene spans 10,898 base pairs and is composed of 10 introns, 11 exons (Opavský et al., 1996) and a single MN promoter (Pastorek et al., 1994). Exons 2-8 code for the CA domain, whilst exons 10 -11 code for the transmembrane cell surface anchor and the intra-cytoplasmic tail (Opavský et al., 1996). Its CA domain contains most of the active site residues, therefore emphasising its catalytic activity (Opavský et al., 1996). The C-terminus and N terminus distinguish CAIX from all other carbonic anhydrases. CAIX displays strong DNA binding capacity, the binding site
probably being located at the N terminus helix-loop-helix, in conjunction with the CA domain.

The MN promoter is located between −173 to +31, with further transcriptionally relevant sites being located within the 3.5Kb upstream sequence. Within the −173 to +31 region, essential regions for transcriptional activity are found, namely protected regions 1 and 2 (PR1 and PR2) to which transcription factor specificity factor 1 (SP1) and activator protein 1 (AP1) appear to bind respectively (Kaluzová et al., 2001). Down-regulation of transcriptional activity has been associated with a silencer element, PR4 (Kaluz et al., 1999).

5.2 FUNCTIONS OF CAIX

CAIX is involved in the generation of an alkali intracellular environment using an ion transport mechanism such as the HCO3⁻/Cl⁻ exchange mechanism (Nogradi, 1998; Opavský et al., 1996; Saarnio et al., 1998; Karhumaa et al., 2001). Ion exchange between bicarbonate and chloride, sodium and hydrogen and ATPase proton pump exclusion, and increases in aquaporin activity on endothelial cells, - which increase vessel permeability - (Ivanov et al., 2001) may contribute in the maintenance of intracellular alkalosis and extracellular acidosis (Wykoff et al., 2000).

The importance of CAIX in tumorigenesis is believed to lie in its regulation of acid-base balance. The acidification of the extracellular matrix by CAIX-driven respiratory mechanism, particularly in poorly angiogenic cells, (as shown by a poor correlation between CAIX and MVD) may confer a survival advantage, as low pH protects
mitochondria from hypoxia enabling cells to survive under oxygen depleted conditions. It has also been postulated that by acidifying the extracellular environment, CAIX may promote survival by inhibiting the synthesis of important cellular immunity proteins. CAIX may participate in abnormal intercellular contact enabling tumour cells to proliferate beyond the normal constraints of contact inhibition. This may be supported by the finding that the CAIX gene and protein expression increase with increasing cellular density in vitro (Lievskovská et al., 1999), and the finding that CAIX is co-expressed with molecules that disrupt cell-cell interactions such as endothelial growth factor receptor (EGFR) and erb proteins, which would increase survival of tumour cells (Giatromanolaki et al., 2001). Once CAIX is released, its effects on the pH of the extracellular matrix may precipitate the release of further angiogenic factors such as Interleukin-8, resulting in a reactive angiogenic process. CAIX expression may be important in tumorigenesis as an adaptation to the hypoxic environment and thus serve as a prognostic indicator if its expression is associated with high risk of disease progression (Wykoff et al., 2001).

The structure, location and distribution of CAIX within cells, support the role of CAIX in acid base balance and in intercellular interactions. A role in cellular interactions may be inferred from the localisation of CAIX to basolateral membranes, the experimental finding that the CA domain in a RPTP-beta receptor located in brain was able to bind contactin, a molecule involved in cellular signalling (Peles 95(Pastoreková et al., 1997), and the in vitro cell density dependent expression of CAIX (Pastorek et al., 1994). Such cellular interactions may occur through the M75 epitope on the proteoglycan domain, since M75 antibody blockades this response, as shown by affinity chromatography (Závada et al., 2000; Opavský et al., 1996).
Evidence suggests that CAIX behaves as an adhesion molecule by its expression at sites of cell-to-cell adhesion in the cellular lateral membrane, which interestingly correlates with the expression of E-cadherin molecules (Svastová et al., 2003). E-cadherin participates in the formation of adherent junctions between epithelial cells by forming complexes with α-catenin and β-catenin. It has been suggested that CAIX may decrease cell-to-cell adhesion by reducing the binding of E-cadherin to β-catenin, though yet to be elucidated pathways. It has further been proposed that CAIX may act as a signal transducer, similar to the actions of EGFR.

5.3 EXPRESSION OF CAIX IN NORMAL TISSUES AND TUMOURS

As briefly mentioned above, the localisation of CAIX is important in order for its functions to be performed.

5.3.1 CAIX in normal tissue

CAIX expression has been found in labile cells with high proliferative capacity. In the human gastrointestinal tract, CAIX has been located in the basolateral plasma membrane of proliferating cryptal enterocytes (the intensity of which decreases along the gut cranio-caudal axis), the pancreatic duct, bile ducts and in gallbladder epithelium (Pastoreková et al., 1997) (Saarnio et al., 1998; Ivanov et al., 2001; Saarnio et al., 2001). Hepatocytes have not been found to express CAIX (Saarnio et al., 2001).
In the human genitourinary tract, CAIX has been detected in basolateral plasma membranes of efferent ducts in human males (Ivanov et al., 2001; Karhumaa et al., 2001), suggesting the involvement of this gene in the acidification of testicular fluid and water absorption by an ion exchange mechanism (Karhumaa et al., 2001).

5.3.2 CAIX in tumours

Two important observations support the hypothesis that CAIX is involved in tumorigenesis, which is not characteristic of the carbonic anhydrase family. Firstly, uncontrolled cell growth following transfection of NIH3T3 cells with the CAIX gene (Pastorek et al., 1994), and secondly, CAIX expression in tumour cells and not in the corresponding normal tissue such as in a variety of epithelial tumours (Ivanov et al., 2001) and renal cell carcinoma (McKieran et al., 1997; Nogradi, 1998).

The increased expression of CAIX (mRNA and protein expression) as compared with expression in the corresponding normal tissue, propose CAIX as a marker of cellular differentiation, an early indicator of malignant potential and indeed facilitator of malignant transformation. Early work in cervical cancer showed increasing levels of expression of MN protein from normal cervical tissue (where MN was located solely in reserve cells) through to cervical carcinomas (Liao et al., 1994). This progressive detection of CAIX with increasing malignant transformation associated with absent expression in the normal tissues has also been observed in oesophageal epithelium, with increased expression in metaplastic and regenerating epithelium (Turner et al., 1997), through the progressive stages of the adenoma-carcinoma cycle in colorectal cancer (Kivelä et al., 2001), including lymph node metastases and one liver metastasis.
Saarnio et al. (1998), in non-small cell lung cancer (NSCLC) where high, predominantly cytoplasmic expression has been associated with carcinoma in situ, microinvasive neoplasia and 80% of NSCLC (Vermyn et al., 1999), and in breast cancer, with weak detection in benign pathology and more extensive detection in malignant disease both by immunohistochemistry and by RT-PCR (albeit with a concordance rate between these two methods of only 69.4%, accounted for by the use of different tissue sections from the same patient) (Bartosová et al., 2002).

In gallbladder malignancy, Saarnio et al found the opposite pattern of expression: all dysplastic lesions expressed CAIX but only 57% of invasive gallbladder lesions suggesting weaker immunoexpression with increasing malignancy. All hepatocellular tumours were negative for CAIX (Saarnio et al., 2001). Patterns of expression in pancreatic lesions were variable, with greatest immunoexpression in mucinous cystadenomas, weaker immunoexpression in benign microcystic adenomas and no immunostaining in endocrine tumours (Kivelä et al., 2000). The statistically significant co-expression of CAIX with a known marker of cellular proliferation, Ki-67, in colorectal tumours (Saarnio et al., 1998) biliary tumours (Saarnio et al., 2001) and hyperplastic and dysplastic lesions of the pancreas (Kivelä et al., 2000) may serve to re-affirm its potential role as a marker of proliferation. Interestingly, in NSCLC (Giatromanolaki et al., 2001), normal cells adjacent to tumour cells also showed immunoreactivity, for reasons yet unclear, a finding also reported in pancreatic lesions (Kivelä et al., 2000), and in breast cancer (Wykoff et al., 2001).

There appears, however, to be no clear explanation for up-regulation of CAIX in cancer. Further genetic analysis of CAIX cDNA in normal tissue (stomach) and
tumorigenic cells (HeLa) revealed no mutational differences (Kaluz et al., 1999; Pastoreková et al., 1997). MN appears to be regulated at the transcriptional level with studies identifying positive and negative regulatory regions in the promoter sequence. This promoter was active in HeLa and MaTu cells. A proximal silencer was identified which is bound by a repressor in order to control MN transcription; therefore, in order for MN to become active this repression must be overcome by processes yet unknown (Kaluz et al., 1999).

5.4 CAIX AND HYPOXIA

Up-regulation of CAIX mRNA and protein expression has been associated with hypoxia (Wykoff et al., 2001), and detected in Von Hippel Lindau-defective cells (Ivanov et al., 2001).

VHL targets HIF-alpha for proteolysis, therefore, defective VHL causes stabilisation of HIF and therefore up-regulation of its activity. Strong CAIX promoter activity occurs in a HIF-dependent manner, (having identified an HRE within the CAIX promoter) (Wykoff et al., 2001).

Northern blot analysis of renal cell cancer lines showed increased CAIX expression in those lines with mutated VHL in association with hypomethylation of CpG sites #1 and #3 in the 5’region of the CAIX gene, whereas those with wild-type VHL expressed little CAIX in association with hypermethylated CAIX gene. However, not all cell lines displayed such up-regulation, implying that other mechanisms are likely
to be involved. Since VHL targets HIF-1α for degradation (Pantouck et al., 2003), the control of CAIX and HIF-1α by VHL are probably inter-related (Ashida et al., 2002).

CAIX gene transcription and protein expression may be regulated by wild type p53 through mediation of HIF-1. Activation of p53 by DNA damage has been shown in vitro to increase HIF-1 degradation with consequent reduction in CAIX protein expression (Kaluzová et al., 2004).

In conditions of high cell density, the PI3K pathway (which also regulates VEGF) has been identified in vitro as regulatory of CAIX expression interdependent to HIF-1α. It has thus been postulated that under hypoxic conditions, HIF is the mayor up-regulator of CAIX whereas in dense cell culture, PI3K is the principle up-regulator, with only a baseline level of HIF being required. This may explain the localisation of CAIX to non-necrotic tumour areas (Kaluz et al., 2002).

5.4.1 CAIX and necrosis

Immunohistochemical analysis of various solid tumours have confirmed significant CAIX expression in focal, peri-necrotic areas, with highest expression closest to areas of necrosis, possibly reflecting the control of CAIX expression by oxygen, also demonstrated in cell culture. This membranous, peri-necrotic distribution has been found in breast cancer (48% in 103 cases of invasive breast cancer (Chia et al., 2001) 59% of ductal carcinoma in-situ (DCIS) and 38% of DCIS and invasive disease Wykoff et al), and in ~90% of head and neck squamous cell carcinoma (HNSCC) tissue samples (Beasley et al., 2001). It was thus postulated that tumour hypoxia
stimulates up-regulation of CAIX, thus explaining its high level of expression in such peri-necrotic areas. Indeed, CAIX was strongly induced \textit{in vitro} and \textit{in vivo} by hypoxia in HNSCC, correlating with oxygen diffusion distances and with microvessel density (Beasley et al., 2001). Giatromanolaki et al. also showed increased expression of CAIX over an increasing oxygen perfusion gradient in NSCLC (Giatromanolaki et al., 2001) and in association of other angiogenesis-related processes: CAIX positively correlated with HIF-1, MVD, EGFR (the latter contrasting Chia et al.'s findings) and PD-ECGF and bFGF pathways. An immunohistochemical analysis of non-metastatic nasopharyngeal carcinomas also correlated CAIX expression with that of HIF1α (Hui et al., 2002); and in another study, CAIX was associated with over-expression of the oncogene c-erbB2 involved in receptor kinase activity, but the functional implication of this finding remains unclear (Bartosová et al., 2002). However, CAIX does not appear to correlate with VEGF expression in any of these studies (Chia et al., 2001; Giatromanolaki et al., 2001; Hui et al., 2002) or in superficial and invasive bladder tumours (Turner et al., 2002), despite their apparently similar tissue distribution. Firstly, CAIX may be a reflection of chronic hypoxia whereas activation of cytokines such as VEGF may be a reflection of acute hypoxia. Secondly, CAIX may also be activated by other cytokines such as bFGF released from apoptotic cells and thirdly, chronic hypoxia and CAIX induction may result if VEGF-driven angiogenesis is not sufficient to sustain tumour growth (Giatromanolaki et al., 2001). This tight regulation enabled CAIX to have a proposed role as a marker of hypoxia, further emphasised by comparable patterns of expression with an established marker, pimonidazole (Ivanov et al., 2001; Wykoff et al., 2001).
Measurements of tumour oxygenation principally in cervical cancer have enabled a more detailed approach to the relationship between CAIX and hypoxia, and the perinecrotic distribution of CAIX. In one study (Loncaster et al., 2001), CAIX expression was found in 94% of prospective locally advanced squamous cell carcinomas cases and 71% of retrospective cases. Hypoxia, (expressed as median partial pressure of oxygen pO2 and percentage of values <5mmHg) was measured by oxygen electrodes in cervical cancer, and high correlation between expression of CAIX and areas of hypoxia was obtained, attributed to the specific localisation of CAIX protein at its site of synthesis. However, a coefficient of 0.5 obtained could be explained by other factors acting on CAIX expression in addition to hypoxia, and the fact that the oxygen measurements were likely to have reflected acute rather than chronic hypoxia which CAIX is believed to represent.

5.5 CAIX: PROGNOSIS

5.5.1 Prognosis

Poor outcome has been correlated with CAIX expression, particularly in association with necrosis, higher tumour grade and increased vascular density in breast cancer (Chia et al., 2001), and high bFGF and high MVD in NSCLC (Giatromanolaki et al., 2001). CAIX has also been associated with poor outcome in cervical cancer, (Loncaster et al., 2001) soft tissue sarcoma (Maseide et al., 2004).
6. EPIDERMAL GROWTH FACTOR RECEPTOR

Protein kinases are enzymes that intricately mediate complex pathways of cellular signalling vital to the control of cell growth and differentiation. These enzymes are classified as either protein-serine/threonine kinases or protein tyrosine kinases. Forty-three tyrosine kinase proteins have been identified, 58 of which are cell receptors. Mutations and over-expression of these receptors have been implicated in tumourigenesis (Roskoski, 2004; Starling and Cunningham, 2004).

Epidermal growth factor receptor (EGFR) is one of the most widely studied proto-oncogene receptors participating in cellular signalling, which precipitates a variety of cellular responses involved in gene transcription and cell growth (Prenzel et al., 2001). The receptor displays high homology to the chicken viral oncogenes v-erbB, first identified in A431 cells (Downward et al., 1984; Hunter, 2002; Carter and Kung, 1994).

6.1 STRUCTURE OF EGFR

The EGFR family (or type 1 receptor tyrosine kinase) derives from the human gene ErbB, so named due to its homology with the avian erythroblastosis viral oncogene v-erbB. It consists of four subtypes that form homo and heterodimers after ligand binding. These subtypes are EGFR/ErbB1, HER2/Neu/ErbB2, HER3/ErbB3 and HER4/ErbB4, all consisting of a ligand-binding extracellular domain, a transmembrane domain and a tyrosine kinase intracellular domain (Roskoski, 2004).
EGFR is a transmembrane, bilobar glycoprotein of 170kDa consisting of 1186 amino acids on one polypeptide chain divided into extracellular, transmembrane, juxta-membrane and protein kinase domains, and a carboxyl terminal region (Carter and Kung, 1994) as depicted in Figure 1.1 [adapted from (Hunter, 84)]. The extracellular component (622 residues) consists of four domains (I-IV) and binds extracellular ligands and activates kinase activity (Carter and Kung, 1994). The transmembrane region consists of 23 amino acid residues, and spans the cell membrane. It is not usually subject to mutational change (Carter and Kung, 1994). The juxta-membrane region separates the membrane region from the protein kinase region and contains residues that undergo phosphorylation by different protein kinases, resulting in either activation or inhibition of receptor function (Carter and Kung, 1994). The protein
kinase domain is structurally constructed in a manner typical to tyrosine kinase receptors. The cleft that separates two lobular subdomains that bind ATP constitutes the active site and regulates the catalytic function of the receptor; hence, any mutations can disable or enhance receptor activity (Carter and Kung, 1994) (Lynch et al., 2004). The carboxyl-terminal region consists of tyrosine phosphorylation sites, and a calcium- modulating caln region that is involved in receptor internalisation (Carter and Kung, 1994; Roskoski, 2004).

6.1.1 Ligands that bind to the EGFR family

Epidermal growth factor, transforming growth factor-α, amphiregulin, betacellulin, neuregulins (NRG) and heparin-binding epidermal-like growth factor are derived from a single glycoprotein precursor by protein cleavage. These factors have one domain in common, consisting of six cysteine residues arranged as three disulphide bridges, which bind to the EGFR. However, different receptors display affinity to different ligands, as shown in Table 1.1, below (Prenzel et al., 2001; Roskoski, 2004).

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Ligand</th>
</tr>
</thead>
<tbody>
<tr>
<td>ErbB1/EGFR</td>
<td>EGF, TGFα, amphiregulin, betacellulin, heparin-binding EGF-like growth factor</td>
</tr>
<tr>
<td>ErbB2/HER2</td>
<td>No activating ligand identified</td>
</tr>
<tr>
<td>ErbB3/HER3</td>
<td>Amphiregulin, NRG 1, NRG2</td>
</tr>
<tr>
<td>ErbB4/HER4</td>
<td>NRG 1-4, EPR</td>
</tr>
</tbody>
</table>
6.2 ACTIVATION AND REGULATION OF EGFR

Receptor activation is initiated by ligand binding to the first and third domains. Ligand binding causes receptor dimerisation /oligomerisation (Yarden and Schlessinger, 1987), either by the formation of inter-receptor disulphide bonds (Gullick, 1994) or by conformational changes (Greenfield et al., 1989; Roskoski, 2004).

This results in the auto-phosphorylation of tyrosine kinase domains in the carboxyl terminus, which potentiates signal transduction through a variety of mediator proteins and culminates in a specific cellular response (Prenzel et al., 2001). The phosphorylation of tyrosine residues creates docking sites at the receptor for a plethora of cytoplasmic mediator proteins (Grb7, Grb2) or enzymes (phospholypase Cγ (PLCγ), phosphatidylinositol 3 kinase and src) which bind through their Src-homology-2 (SH 2) or phosphotyrosine-binding (PTB) domains and serve to induce or amplify the associated intracellular signal cascade(s) (Roskoski, 2004; Brown, 1995; Prenzel et al., 2001) with the aid of scaffold proteins (Hackel et al., 1999). In addition to ligand-dependent EGFR activation, EGFR can be activated by ligand-independent mechanisms, or transactivation (Hackel et al., 1999) (Figure 1.2).

Following activation and initiation of the cellular response, the EGFR receptor is either recycled or degraded. Ligands target the receptor/growth factor complex for lysosomal degradation in a process called ligand-induced endocytosis (Roskoski, 2004). Once inside the lysosome, most complexes dissociate allowing for receptor recycling, except for the EGF/EGFR complex, which results in the ubiquitinisation
(by ubiquitin-protein ligase c-Cbl), and degradation of the EGFR. Threonine phosphorylation of the C terminus of EGFR, however, can prevent ubiquitinisation and hence enable receptor recycling (Prenzel et al., 2001).

6.3 FUNCTIONS OF EGFR

Numerous biochemical intracellular signalling pathways are initiated upon activation of the EGFR, and most remain to be fully elucidated. The principal roles of EGFR appear to involve control of cellular proliferation, mediation of gene transcription, promotion of cell survival, modulation of cellular adhesion, and induction of angiogenesis. Figure 1.2 illustrates the interactions between these intracellular signalling pathways [adapted from (de Bono and Rowinsky, 2002)].
EGFR

Tyrosine

Phosphorylated tyrosine

Grb-2

Famesyltransferase

P13 Kinase

SOS

RAS

Raf

MEK

Pro-Ras

Rac

MEK

SEK

Rho

ERK

JNK

Cytoskeletal Organisation

Gene Activation
Cell Cycle Progression

Angiogenesis/Metastases

Proliferation

Apoptosis

Differentiation

Nucleus
Cellular proliferation is controlled by the MAP pathway. Following activation of the EGFR receptor, a guanine nucleotide releasing factor, or sos complex is formed which activates G protein ras at the plasma membrane. Following a series of phosphorylative reactions, protein kinases MEK and MAKP are activated which regulate transcription factors such as extracellular regulated kinases (ERKs) (Brown, 1995; Prenzel et al., 2001; Roskoski, 2004).

Gene transcription is mediated by Signal Transducers and Activators of Transcription (STATS) (Andl et al., 2004; Brown, 1995; Prenzel et al., 2001). Activated EGFR promotes the phosphorylation (via Jak dependent or independent pathways), dimerisation and relocation of STATS from the cell membrane to the nucleus, where they bind to the specific response elements of target genes involved in cellular proliferation.

Activated EGFR also promotes the phosphorylation of phosphatidylinositol-3-kinase (PI3K) with subsequent downstream activation of Protein kinase B (Akt) which has an important role in cell survival by activating anti-apoptotic factors, thus allowing cells to progress through the cell cycle. This pathway also appears to be important in the GTPase-controlled organisation of the actin cytoskeleton, particularly in relation to cell spreading and migration (Marcoux and Vuori, 2003).

Cell-to-cell, cell-to-extracellular matrix and cadherin-dependent cell adhesions are mediated by growth factor receptors including EGFR and the integrins (Prenzel et al., 2001; Barbosa et al., 2003).
6.4 EXPRESSION OF EGFR IN NORMAL TISSUE AND TUMOUR

Abnormal activation of EGFR by receptor mutation, over-expression with or without gene amplification and deranged regulatory control mechanisms have been described in various solid tumours and are targets for developing chemotherapeutic agents.

6.4.1 Receptor mutations

Somatic mutations or chromosomal changes in protein kinase receptors predispose to tumour formation. Mutations of both the serine/threonine kinase gene (BRAF) and the tyrosine kinase genes have been identified. Deletions in the intra-cellular and extra-cellular domains of the EGFR receptor have been described in Table 1.2 (Voldborg et al., 1997; Lynch et al., 2004; Paez et al., 2004).

Two important studies have very recently evaluated EGFR mutations in patients with NSCLC, with particular reference to patients demonstrating a good clinical response to gefitinib (Iressa, Astra Zeneca), a tyrosine kinase inhibitor which targets the ATP cleft in the receptor. Most patients do not respond well to treatment with this agent, (Lynch et al., 2004) however, a small subgroup of patients have shown a remarkable response, to the extent that this drug was recently licensed in the USA and Japan for the treatment of patients with disease refractory to conventional chemotherapy.
Table 1.2 EGFR mutations in solid tumours

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Description</th>
<th>Tumour</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFRvI:</td>
<td>Deletion of the extracellular domain to resemble the oncogene v-erb-B, rendering the receptor unable to transmit signals in response to EGF</td>
<td>Malignant glioma</td>
</tr>
<tr>
<td>EGFRvII:</td>
<td>Deletion of 83 amino acids between the transmembrane and the ligand binding domain, retaining its capacity to transmit signals upon activation</td>
<td>Gliomas</td>
</tr>
<tr>
<td>EGFRvIII:</td>
<td>Amino acids 6-273 of the extracellular domain are deleted and replaced by a glycine residue, forming a truncated, mutant receptor. It stimulates cell proliferation independent of ligand binding by a persistently active tyrosine kinase function and is over-expressed at the cell membrane</td>
<td>Ovarian cancer, non-small cell lung cancer, gliomas, breast cancer.</td>
</tr>
<tr>
<td>Cytoplasmic domain</td>
<td>Deletions in domains regulating EGFR degradation, leading to overexpression being documented.</td>
<td>Glioblastomas</td>
</tr>
<tr>
<td>Deletion mutations</td>
<td>In-frame deletions of amino acids 746-750, 747-751, 747-753 leading to clinical response to tyrosine kinase inhibitor gefitinib</td>
<td>NSCLC</td>
</tr>
<tr>
<td>Substitution mutations</td>
<td>Amino acid substitution G for T (L858R), A for T (L861Q), T for G (G719C) leading to clinical response to tyrosine kinase inhibitor gefitinib</td>
<td>NSCLC</td>
</tr>
</tbody>
</table>

It was found that receptor mutations identified in this small cohort of patients were associated with this remarkable clinical improvement. It was further postulated that receptor mutations rather than non-mutated receptor over-expression may be important in certain groups of patients, which upon identification could facilitate targeted therapy.
In a series of 119 primary NSCLC tumours from the USA and Japan, Paez et al (Paez et al., 2004) identified substitution mutations, (leucine-858 to arginine) and deletion mutations in the kinase domain of 16 tumours. The substitution mutations were located in the activation loop and the trisphosphate-binding domain, both important for kinase autoregulation. Normal lung tissue had wild-type receptor genes. Interestingly, mutations were more common in adenocarcinomas, and in female patients of Japanese origin. It was very importantly and interestingly found that patients with a good clinical response to gefitinib (5/125 patients treated in the USA) had mutations to the EGFR kinase domain, in contrast to those with poor clinical response (4/125 patients) (p=0.0027). In vitro work showed impaired EGFR autophosphorylation in cell lines with substitution mutations treated with 100nM gefitinib, whereas cell lines without mutations required 100x higher drug concentrations to achieve the same response.

Lynch et al (Lynch et al., 2004) also identified receptor mutations in a small cohort of patients with disease refractory to standard chemotherapy but clinically responsive to gefitinib. Nine tumours were analysed from 25 patients treated (tumour availability). Eight displayed heterozygous mutations in the tyrosine kinase domain. No mutations were detected in seven patients who did not respond to gefitinib treatment (p=<0.001). The group proceeded to examine a further 25 NSCLC tumours, 95 other primary tumours and 108 cell lines. EGFR mutations were only detected in two NSCLC specimens. In vitro work showed that mutant receptor phosphorylation was greater and remained so for longer with the addition of EGF as compared with wild-type receptors.
6.4.2 EGFR in normal tissue

EGFR is an important constitutive component of normal human tissue both during embryogenesis and in mature tissue (Damjanov et al., 1986). The receptor has been immunohistochemically located, in a linear or granular pattern of reactivity, to the cell surface of proliferating epithelia (Damjanov et al., 1986) such as in normal breast ducts and acini (Fox and Harris, 1997; Damjanov et al., 1986), prostate (reviewed Damjanov et al., 1986) and basal cells in squamous cell epithelium, where it has been found to be essential in the development of normal glandular tissue and for normal physiological functions (Fox and Harris, 1997). EGFR has also been described in the cytoplasm of most cellular components of tissues such as liver, prostate and pancreas (Damjanov et al., 1986).

The different localisation patterns of expression have been related to the status of EGFR: cytoplasmic location may represent receptor-ligand complex internalisation prior to degradation; membranous expression suggests receptor-ligand complex localised to the cell membrane (Karameris et al., 1993; Koretz et al., 1990).

6.4.3 EGFR in solid tumours

Mutant EGFR, EGFRvIII, is not found in normal tissue (Voldborg et al., 1997). Cell culture models suggest that mutated EGFR potentiates tumour invasion by up-regulating genes involved in the formation and breakdown of the extracellular matrix,
such as collagens and fibrillins, and matrix metalloproteinases respectively (Cox et al., 2000; Lal et al., 2002).

High expression of EGFR has been found in a range of solid tumours including oral (Todd and Wong, 1999), gastric (Yasui et al., 1988), oesophageal (Mukaida et al., 1991), head and neck (Putti et al., 2002), transitional cell carcinoma of the bladder (Neal et al., 1985), breast (Aziz et al., 2002; Fox and Harris, 1997), pancreatic cancer and non-small cell lung cancer (Cox et al., 2000). Tumour differentiation and malignancy/invasion has been described in oesophageal (Mukaida et al., 1991), gastric (Yasui et al., 1988), head and neck squamous cell carcinoma (Putti et al., 2002), and transitional cell carcinoma of the bladder (Neal et al., 1985) although these observations are by no means consistent in all studies (Christensen, 1998).

Mechanisms to explain increased levels of EGFR in tumours have been proposed using prostate cancer cell lines (Kim et al., 1999), and in colorectal and pancreatic tumours (Yamanaka et al., 1993). An autocrine growth factor loop is believed to stimulate EGFR and TGFα, facilitated by the loss of cellular morphological organisation (typical of deranged cell growth in tumorigenesis); this appears to allow the normally apically located EGF to bind to the basolaterally located receptor. Up-regulation of the receptor promotes further growth factor production such as TGFα, thus positively reinforcing the autocrine loop, as concomitant expression of TGFα mRNA and EGF mRNA has been identified in colorectal tumours (Ito et al., 1990).
6.5 EGFR AND HYPOXIA

Hypoxia, as detailed above, promotes the up-regulation of genes involved in tumourigenesis. EGFR has also been found to be up-regulated by hypoxia, through a growth factor called early growth response-1 (egr-1), which has also been implicated in the up-regulation of other genes involved in responses to cellular stress. Nishi et al demonstrated marked up-regulation of the EGFR gene by Egr-1 in vitro, (a binding site for Egr-1 on the EGFR promoter was also found) using U-2OS human osteosarcoma cell lines (Nishi et al., 2002).

6.6 EGFR IN COLORECTAL CANCER

The clinical significance of EGFR expression in colorectal tumours is unclear. Various studies examining EGFR expression and its relationship to clinicopathological variables, tumour invasion and differentiation have yielded contradictory results possibly due to the methods of detection and sensitivity of the method utilised, and the inconsistencies in detection even within a single series. (Steele et al., 1990; Steele et al., 1990; Karameris et al., 1993; Yasui et al., 1988; Koretz et al., 1990).

EGFR has been proposed as a marker of tumour differentiation by studies using northern blot analysis (Ito et al., 1990), indirect immunofluorescence, western transfer and EGF binding studies (Bradley et al., 1986; Hunter, 2002), and immunohistochemistry (Steele et al., 1990). Higher levels of EGFR mRNA in colonic tumours compared with normal colonic mucosa (Ito et al., 1990), higher EGFR
expression in moderate to well-differentiated carcinomas in comparison to poorly
differentiated carcinomas and normal colonic tissue (Bradley et al., 1986; Hunter,
2002), and greater cytoplasmic detection of EGFR by immunohistochemistry in
poorly differentiated tumours (although the degree of staining however varied
according to the immunohistochemical visualisation technique used) (Steele et al.,
1990).

Other studies failed to demonstrate any significant correlation between EGFR
expression, tumour invasion and differentiation. Immunohistochemistry, western
blotting and iodine-labelled EGF binding analysis demonstrated expression in 77.1%
of 61 tumours analysed, but no statistical correlation to tumour characteristics (Yasui
et al., 1988). In a larger series of 44 normal colon tissue specimens (adjacent/distant
from the tumour), 25 adenomas and 144 adenocarcinomas, no clear association
between EGFR expression and type of tissue was obtained, with intense staining only
detected in 11 carcinoma cases. Correlation with clinicopathological parameters did
not reach statistical significance, thus the authors concluded that EGFR expression in
colorectal cancer is complex and may reflect individual patients’ genetic constitution
(Koretz et al., 1990).

6.7 EGFR - PROGNOSIS AND POTENTIAL THERAPEUTIC TARGET

6.7.1 Prognosis

EGFR has been correlated with poor prognosis in oesophageal (Mukaida et al., 1991)
and breast cancer (Aziz et al., 2002), particularly in association with high tumour
vascularity in node negative patients (Fox and Harris, 1997), although oestrogen receptor positive tumours express significantly less EGFR compared with oestrogen receptor negative tumours (Sainsbury et al., 1985). In oral cancers, the prognostic significance remains unclear (Todd and Wong, 1999). Co-expression of EGFR and CAIX was associated with a worse prognosis compared with that of either factor alone in a recent study by Swinson et al. This suggests that EGFR may enhance the expression of CAIX, act as a survival factor for cells growing in a hypoxic environment, or both (Swinson et al ASCO 2002, unpublished data).

Experimental animal models have been used to examine the role of EGFR in the metastatic potential of colorectal cell lines. Cultured cells derived from increasing Dukes’ stage tumours were injected into the spleen of nude mice, with subsequent analysis of the liver metastases produced. EGFR mRNA and protein expression were found to be higher in cells with high metastatic potential compared to those with low metastatic potential, and were associated with more copies of chromosome 7, thought to be involved in metastatic behaviour (Radinsky et al., 1995). EGFR may contribute to the establishment of a metastatic deposit upon activation by TGF-α, which is normally produced by hepatocytes to promote repair mechanisms. Upon malignant cell “soiling”, proliferative repair mechanisms may be activated in normal liver tissue, which may also have the effect of stimulating the proliferation of metastatic cells with high EGFR expression (Radinsky et al., 1995).

Contrary to a large proportion of solid tumours (detailed above), the prognostic value of EGFR expression in colorectal cancer has not been widely studied. Only two series has correlated increased EGFR expression in colorectal tumours with poor survival
Increased EGFR expression has been correlated to poor patient survival. In one series of 82 colorectal tumours (Mayer et al., 1992), 80/82 colorectal tumours expressed EGFR, with 51 cases displaying maximal intensity by immunohistochemical analysis. The percentage tumour staining was found to be significantly associated with worse prognosis; >50\% expression was associated with shorter survival as compared with <50\% expression. In a second study, elevated EGFR levels, as determined by iodine-125-labelling, were associated with poor prognosis in patients with colorectal cancers (p=0.024) and rectal cancer (p=0.002). Indeed, high EGFR was found to be independently prognostic on multivariate analysis (Reinhard et al., 2003).

7. THE METALLOPROTEINASES

In order for malignant cells to initiate the metastatic cascade and intravasate into blood vessels, they must first break down the type IV collagen basement membrane and the extracellular matrix. This process is believed to occur through alterations in cell-to-cell and cell-to-matrix adhesions, and by the infiltration of the basement membrane with the aid of specific enzymes called matrix metalloproteinases (MMPs), (reviewed by Cox et al., 1999; Chambers and Matrisian, 1997) as evidenced by inverse immunoreactivity between active MMP-9 and type IV collagen in a study of 34 colorectal tumours (Zeng et al., 1999).
7.1 STRUCTURE OF THE METALLOPROTEINASES (MMP)

Metalloproteinases are classified according to their substrate specificity, which is determined by differences between the catalytic domains. The principal types of MMP are tabulated below.

Table 1.3 Classification of the Metalloproteinases

<table>
<thead>
<tr>
<th>MMP</th>
<th>Type of enzyme</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>1, 8 and 13</td>
<td>Collagenases</td>
<td>To degrade fibrillar collagens</td>
</tr>
<tr>
<td>3, 7 and 10</td>
<td>Stromelysins</td>
<td>To target proteoglycans, fibronectin, laminin and type IV collagens</td>
</tr>
<tr>
<td>2 and 9</td>
<td>Gelatinases A and B</td>
<td>To degrade gelatin and type I and IV collagen and type IV and type V collagen respectively</td>
</tr>
<tr>
<td>12, 18 and 19</td>
<td>Elastases</td>
<td>To target elastin</td>
</tr>
</tbody>
</table>

More novel MMPs, which can be either membrane bound (MMP-14, MMP-15, MMP-16 and MMP-17) or secreted as an activated enzyme (MMP-11) contain an extra protease recognition sequence (RXKR), and also degrade the principal components of the extracellular matrix (Cox et al., 1999).

The gelatinases specifically were included in this study due to their possible correlation with angiogenesis (Kim and Kim, 1999), and their role in tumour invasion in colorectal liver metastases (Zeng and Guillem, 1995).
7.2 ACTIVATION AND REGULATION OF THE METALLOPROTEINASES

7.2.1 General principles

The regulation of the MMPs relies on the activation of their latent pro-enzyme forms, promotion of activity by pro-angiogenic growth factors and suppression of activity by endogenous tissue inhibitors of metalloproteinases (TIMPs).

MMPs are zinc endopeptidases secreted in a pro-enzyme form by stromal cells, fibroblasts and endothelial cells in proximity to tumour tissue. Pro-enzyme activation is believed to occur either when pro-MMPs (latent enzymes) bind with membrane-bound MMPs (Zeng et al., 1999), by plasmin-facilitated cleavage of an N-terminal sequence (thus indirectly influenced by the plasmin cascade) and alterations in the bond between the constituent zinc atom and the pro-enzyme domain (PRCGVPD), (Chambers and Matrisian, 1997) (Cox et al., 1999) (Gu et al., 2002) or by cell mediated mechanisms.

MMPs are up-regulated by pro-angiogenic growth factors, such as EGF, basic fibroblast growth factor, interleukin 1β, and tumour necrosis factor, and by endogenous ERK signalling pathways, particularly involving integrin αvβ6 (inhibition of this integrin suppresses MMP-9 expression in vitro) (Gu et al., 2002). Thrombospondin-1 is also believed to activate MMP-9 activity, as in a series of 99 colorectal carcinomas, there was a positive correlation between protein expression of MMP-9, TSP-1 and its receptor (Wakiyama et al., 2001).
The stromal TIMPs (TIMP-1 and TIMP-2), secreted by vascular smooth muscle, endothelial cells and fibroblasts in tumour tissue, form complexes with the activated MMPs to down-regulate their activity (Joo et al., 1999).

### 7.2.2 Effect of micro-environmental acidity on MMP production

A variety of *in vitro* studies have examined the effects of extracellular pH on MMPs (below). Production and activity are typically measured by rates of tracer incorporation into the constituent matrix, fluorescent dye assays and gelatin zymography. Invasive potential is assessed using a membrane invasion culture system (MICS) assay. As indicated below, the principal findings demonstrate that an acidic pH is required for optimum activity, but with differential sensitivities according to the type of tissue and the MMP examined.

In articular chondrocytes and nucleus pulposus disc cells (Razaq et al., 2003), maximal total MMP production was detected at pH 7.2, but sharply declined with further acidification (pH 6.4). The effects of acidification on activity were more apparent in chondrocytes than in nucleus pulposus disc cells possibly because chondrocyte-associated MMP-2 seems to be more pH sensitive than MMP-9 found in discs. Indeed, acidic conditions have been shown to promote gelatinolytic activity in mouse B16-F10 melanoma cells (acidic media was established using ammonium sulphate) (Kato et al., 1996). In this experiment, increased expression of MMP-9 mRNA was detected at pH 5.9 than at pH 7.3, due to increased gene expression rather than cytokine-mediated up-regulation (Kato et al., 1996).
Increased activity in association with acidification of the microenvironment has also described in poorly invasive A375P and highly invasive C8161 human melanoma cells. An extracellular pH of 6.8 was more conducive to the more invasive phenotype, reflected by higher invasion rates detected in the study. Cells grown for three weeks at pH 6.8 were found to display greater invasion through the basement membrane than control cells (grown at pH 7.4), and have increased amounts of active MMP-9 and pro-MMP-2. The authors thus postulated that acidic conditions were conducive to a more invasive phenotype due to the activation of certain gene products such as the metalloproteinases (Martínez-Zaguilán et al., 1996).

7.3 MMPS IN COLORECTAL CANCER

7.3.1 Localisation of MMP-2 and MMP-9

Metalloproteinases have been detected in a variety of solid tumours, including prostate, breast, gastric (Parsons et al., 1998), hepatocellular (Ahida et al., 1996), colorectal (Zeng et al., 1999), and non-small cell lung cancer (Cox et al., 2000).

The precise localisation of MMPs in colorectal cancer and the surrounding tissue is not consistent across the literature. Results vary between in vitro and in vivo cell line work, and studies using surgically resected colorectal specimens. MMP-2 and MMP-9 have consistently been found in the stroma adjacent to the tumour (Pyke et al., 1993; Poulson et al., 1992), particularly in infiltrating inflammatory cells (Zeng and Guillem, 1996; Wakiyama et al., 2001; Collins et al., 2001) suggesting that MMP expression is associated with inflammatory infiltration as opposed to being an
inherent biological characteristic of the tumour. However, stromal MMP-2 and-9 have not been detected by immunohistochemistry (Kim and Kim, 1999) or zymography (Parsons et al., 1998) on surgically resected specimens. Furthermore, in the latter study, no correlation was found between MMP expression and lymphocytic infiltration as evaluated by the Jass classification (Parsons et al., 1998).

Equally, MMP localisation to the tumour cells is conflicting. Some authors have failed to demonstrate \textit{in vitro} MMP-2 and MMP-9 expression in human tumour cell lines (McDonnell et al., 1999; Zeng and Guillem, 1996; Zeng and Guillem, 1995), whereas others have clearly localised MMP-2 and MMP-9 \textit{in vitro} (Nakajima et al., 1990) and more specifically to the cell cytoplasm by immunohistochemical analysis. (Wakiyama et al., 2001; Kim and Kim, 1999) Kim and Kim argue that zymography cannot accurately detect cellular localisation, which was used with the cell lines.

\textbf{7.3.2 Expression of MMPs and tumour stage}

The relationship between the expression of MMPs and disease progression has been evaluated in a number of studies with varying results, particularly when active versus latent forms of different MMPs were considered.

Gene expression of MMP-2 and MMP-9 using real time PCR in surgically resected normal colorectal tissue, adenomas and carcinomas (Heslin et al., 2001) demonstrated a significant increase in gene expression of MMP 7 isolated in adenomas and carcinomas as compared with normal tissue. Gene expression of MMP-2 and MMP-9 was only significantly higher in carcinomas as compared with normal tissue; none
was localised in adenomas. The authors surmised that MMP-7 up-regulation seems to occur early in the adenoma carcinoma cycle, whereas MMP-2 and MMP-9 appear to be up-regulated later in the disease process, possibly in association with invasion (Heslin et al., 2001). Other studies support this theory. Newell et al (Newell et al., 1994) identified MMP-7 expression specific to the cell surface, which would enable tumour cells to disrupt normal cell-cell adhesion, consistent with the first stages of tumour progression. Waas (Waas et al., 2002), Baker (Baker et al., 2000) and Masuda, in three separate studies comparing normal tissue with colorectal tumours (n=73, n=46, n=59 respectively) identified increased MMP-2 and-9 expression, mostly active form, in malignant tissue, particularly at the tumour edge. Waas also noted increased latent forms of both enzymes.

In keeping with the observation that MMPs and MMP activation are needed for tumour invasion, latent enzyme forms have been detected in advanced disease and metastatic phenotypes have been associated with increased activated MMP-2 and MMP-9 (Zeng et al., 1999). Furthermore, it has been shown that the ratio between active and latent MMP-2 may be markedly increased in tumour tissue (Waas et al., 2002). Of interest is the observation that in tumour tissue the levels of pro-MMP-9 were higher than active MMP-9. In Parson et al’s study of 53 patients (Parsons et al., 1998), MMP-2 and MMP-9 were also significantly higher in carcinomas, with increased active to latent ratios. The study also detected pro-MMP-9 in adenomas at a statistically significant higher level than in normal tissue, in contrast to Heslin’s results (Heslin et al., 2001), wherein no pro-MMP-9 was detected in adenomas. Zeng (Zeng et al., 1999) also found latent MMP-2 and -9 in adenomas, but not the active form.
Further evidence is presented by Kim and Kim (Kim and Kim, 1999) who in an immunohistochemical study of 72 patients showed a statistically significant increase in MMP-2 expression at the invasive edge with increasing tumour stage, and a similar trend for MMP-9, although co-expression of these enzymes was not demonstrated. Interestingly, increased expression was noted in well differentiated as opposed to poorly differentiated tumours (Kim and Kim, 1999).

Significantly lower levels of active and latent MMP-2 and a trend towards lower MMP-9 were noted in Waas’ study in advanced stage disease (notably between stage III and IV disease) and in patients with synchronous liver metastases (Waas et al., 2002), but no differences were identified between T stages. The reasons for decreased MMP with advancing stage could be as a result of differences in stromal constituents between stages (Liabakk et al., 1996), MMP involvement in the host’s response to tumour mechanisms, or because slowly growing, less aggressive and by inference less invasive tumours may have more time to up-regulate MMP-9 in contrast to fast growing tumours (Takeha et al., 1997). Zeng et al (Zeng et al., 1996) however, did describe a statistically greater ratio of MMP-9 to normal mucosa expression with increasing Dukes’ stage in carcinomas. Although Collins et al similarly noted higher MMP-2 expression in Dukes’ C tumours compared with B tumours and normal tissue (Collins et al., 2001), they did not detect increased MMP-9 in tumours as compared with normal tissue. Parsons did not find a correlation between MMP expression and any clinicopathological factor evaluated (Parsons et al., 1998).
Other, smaller studies have also described increased MMP-2 and MMP-9 in malignant colorectal cancer. Roeb et al, who evaluated 20 colon cancers and 10 rectal cancers, described increased MMP-9 and MMP-2 enzymatic activity by zymography in colon cancers as compared with normal tissue. Interestingly, however, they did not describe such increases in malignant rectal tissue as compared with their normal counterpart (Roeb et al., 2001). In summary the data indicate that MMP-2 and -9 are detectable and upregulated in malignant as compared to normal tissues but the clinical relevance of this expression requires further clarification.

7.3.3. MMPs in the primary tumour and metastatic disease

Experiments using human colorectal cell lines implanted into nude mice have demonstrated that tumours can induce MMP production in host tissue. McDonnell et al generated in vivo subcutaneous tumours from cell lines of differing metastatic capacity, and detected MMP-2 and MMP-9 expression by gelatin zymography, with greater expression of MMP-9 associated with the more metastatic cell line (McDonnell et al., 1999). These MMPs were not detected in the non-implanted in vitro cell lines. A notable increase in MMP-2 and -9 expression was detected by subsequent implantation of these subcutaneous tumours into the caecum, with concomitant upregulation of other MMPs. Further analysis demonstrated the up-regulated MMP to be of mouse rather than human origin and localised to stromal rather than tumour cells. Nakajima et al produced similar results (Nakajima et al., 1990). Some clinical support to this theory was found by Masuda et al who in a study of 59 patients with colorectal cancer, (with metastases n=13, without metastases n=46) showed that those patients with liver metastases had higher levels of MMP-2.
compared with patients without metastases. They postulated that normal tissue in these patients was at greater risk from tumour invasion due to the presence of increased levels of MMPs to degrade the ECM (Masuda and Aoki, 1999). However, the application of these findings to actual human colorectal cancer is unclear, as these data conflict with the findings of Kim and Kim, who detected only tumour, not stromal MMP-2 and MMP-9 in their series of resected colorectal specimens.

Increased expression in primary tumours has been correlated with synchronous distant metastases and reduced disease-free survival (Collins et al., 2001). Matsuyama et al also showed greater expression of MMP-2 and MMP-9 in primary colonic tumours of patients with liver metastases as compared with those without liver metastases, however only 8 samples (4 from patients with metastases and 4 without) were included. In addition, tumour was collected from different sites: 6 from the primary tumour, one from a lymph node and one from a liver metastasis, and therefore any environmental effects on MMP up-regulation cannot be accounted for (Matsuyama et al., 2002).

MMPs as predictors of disease progression to the liver have been postulated by analysing levels of MMP-2 and MMP-9 in serum and bile from patients with colorectal cancer. Okada et al (Okada et al., 2001) measured MMP levels by zymography in bile of 53 patients with colorectal cancer and found a statistically higher expression of active MMP-2 and pro-MMP-9 in patients with metastatic disease (n=27) compared with normal controls and patients with Dukes’ A-C disease (Okada et al., 2001). No difference in the synchronicity of liver disease was observed. In contrast, Mukai et al failed to show a relationship between MMP-2 in sera and the
presence of liver metastases (Mukai et al., 1999) however, these measurements pertain to patients at one point in their disease process. It would have been interesting, and more useful as a predictor of metastatic disease, to obtain serial measurements by following-up patients over time, to assess the point at which levels of MMPs are upregulated.

7.3.4 MMPs in colorectal liver metastases

Data regarding the expression of MMPs in colorectal liver metastases has been published by Zeng’s group from the Memorial Sloan-Kettering Cancer Centre, and more recently in a study by Waas et al from the Netherlands. In the first study by Zeng, MMP-9 was localised by in situ hybridisation to peri-tumour stroma cells, notably macrophages, at the tumour-normal liver edge (Zeng and Guillem, 1995). In a further study the invading tumour edge contained increased levels of both mRNA and protein expression of MMP-9 (Zeng and Guillem, 1996). Gelatin zymography identified both latent and active forms of MMP-9 in 18 liver metastases, whereas only the latent form was identified in normal liver tissue (Zeng and Guillem, 1998). These observations support the notion that MMP-9 has an important role in tumour invasion and may be controlled by interactions between tumour and stroma cells.

Waas et al examined MMP-9 and MMP-2 activity in 32 colorectal liver metastases, and showed higher latent to active enzyme ratios in metastatic as compared with normal tissue. Both enzymes were associated with early recurrence. Low MMP-2 levels correlated with larger metastases (Waas et al., 2003).
7.4 MMPs AND ANGIOGENESIS

The role of MMPs in angiogenesis is not clear. Hiraoka et al postulate that MMPs contribute to neovascularisation by degrading pericellular fibrin (Hiroaka et al., 1998). Kim and Kim noted a trend towards increased angiogenesis as measured by MVD in association with MMP-2 and MMP-9 (Kim and Kim, 1999). These enzymes are also believed to facilitate angiogenesis (Heslin et al., 2001).

8. HYPOXIA INDUCIBLE FACTOR -1

Hypoxia-inducible factor -1 is ubiquitously expressed in most tissues, which supports its role in physiological responses to hypoxia (Semenza et al., 1997). HIF-1α is the only subunit found in hypoxic conditions, levels being increased by inactivation of tumour suppressor genes and activation of oncogenes (Blancher and Harris, 1998; Richard et al., 1999; Semenza, 2000). HIF-1 has been localised by immunofluorescence microscopy and digital image analysis within very close proximity to blood vessels in cervical cancer xenographs (Vukovic et al., 2001) (peak expression at 0-138 micrometers; decline >204micrometers) with statistically significant correlation with a known chemical marker of hypoxia nitroimidazole.

8.1. STRUCTURE OF HIF-1

Hypoxia inducible factor-1 is a heterodimer consisting of an alpha and a beta subunit (Semenza and Wang, 1992) as determined by protein purification and cDNA sequence analysis (Semenza and Wang, 1992; Semenza et al., 1997). The alpha
subunit consists of 826 amino acids derived from chromosome 14 and the beta subunit is a product of the aryl receptor nuclear translocator gene (ARNT) derived from chromosome 1.

HIF-1 is formed when HIF-1α dimerises with ARNT under hypoxic conditions. (Semenza et al., 1997) HIF-1 contains an N-terminal basic helix-loop-helix domain (bHLH), a period-ARNT-similar (PAS) domain, an oxygen dependent degradation domain, a transactivation domain (amino acids 531-575 and 786-826) (Richard et al., 1999; Semenza, 1998) and an inhibitory domain (amino acids 576-785) (Semenza, 2000). The basic helix-loop-helix domain dimerises by aligning basic residues to create a DNA domain which binds to the hypoxic response element (HRE) of target genes - 50 or less constant core base pair sequences located in the 3’ flanking region, identified as 5’-CGTG-3’ (Semenza et al., 1997). This hypoxia response element was first described in the human erythropoietin gene and its importance in cellular response to hypoxia was postulated upon inhibition of the hypoxic response in association with mutations to this sequence (Semenza et al., 1997; Semenza, 1998). Moreover, the mutations confirmed that HIF-HRE binding is sequence-specific (Semenza et al., 1997).

8.2. REGULATION AND ACTIVITY OF HIF-1

8.2.1 Oxygen-dependent regulation

Although HIF-1 mRNA exists in a steady state in a large number of cells, HIF-1α is not detected in the vast majority of normoxic cells, except, as recently described, in
pancreatic cancer cell lines and in the PC-3 prostate cancer cell line (Akakura et al., 2001). However, under hypoxic conditions, expression is markedly upregulated and localised to the cell nucleus (Richard et al., 1999; Semenza and Wang, 1992; Semenza, 1998; Shih and Claffey, 1998), as demonstrated during experiments with the erythropoietin gene, and in mouse epidermal keratinocytes (Elson et al., 2000).

Immunohistochemical studies have detected HIF-1 both in the nucleus as expected, and in the cytoplasm, suggesting that HIF-1 is made and degraded in the cytoplasm (Giatromanolaki et al., 2001). HIF-1 has also been located in macrophages exposed to hypoxic conditions (Burke et al., 2002).

The levels of HIF-1 protein present and the activity of the transactivation domains in determining the activity of HIF are oxygen-dependent (Semenza, 1998). Expression of HIF-1 in peri-necrotic areas and in the invasive front of tumours, as shown in brain tumours may show that HIF-1 expression relies on different oxygen concentrations (Giatromanolaki et al., 2001). Under normal oxygen tension, HIF-1 has a very short half-life (Richard et al., 1999; Semenza, 1998; Shih and Claffey, 1998). Once formed, degradation occurs via an oxygen-dependent ubiquitin proteosome pathway with the assistance of the Von Hippel Lindau (VHL) tumour suppressor gene. A proline residue, Pro564, located on HIF-1α is hydroxylated by HIF1α-prolyl-4-hydroxylase, and targeted for degradation by binding with pVHL (Brahimi-Horn et al., 2001).

Hypoxic conditions and an inactive VHL gene thus appear to inhibit the degradation of HIF-1α. (Semenza et al., 2000) by (Akakura et al., 2001; Blancher and Harris, 1998; Richard et al., 1999; Semenza et al., 2000). Under these conditions, HIF-1α
dimerises with the aryl hydrocarbon nuclear translocator (Blancher and Harris, 1998) to form a stable complex. p42/p44 MAK phosphorylation pathways may facilitate further complex activation. The complex subsequently translocates to the cell nucleus, where, upon interacting with mediator molecules such as acetyl transferase proteins CBP/p300 (cAMP-responsive element binding-1 binding protein), up-regulates the transcription of pro-angiogenic factors (Blancher and Harris, 1998; Richard et al., 1999).

The relationship between oxygen tension and the expression of HIF-α 1 has been demonstrated in vitro. Decreasing oxygen concentrations were associated with an exponential rise in HIF-1α expression, with maximal response at 0.5% oxygen (Jiang et al., 1996). In vivo, rats exposed to low oxygen concentrations showed increased expression of HIF-α 1 mRNA (Semenza et al., 1997).

Although the exact pathways by which oxygen sensing and gene up-regulation remain unclear, a variety of possible mechanisms have been postulated. Evidence from experiments with the EPO gene suggests that a heme flavo-oxido-reductase protein is involved (Blancher and Harris, 1998; Richard et al., 1999; Semenza, 1998). It is believed that low oxygen concentrations distort the heme molecule between oxy and deoxy states, which initiates a cascade of events that induces HIF –1α (Richard et al., 1999). It has been found that substances that inhibit heme synthesis, such as carbon monoxide or iron chelators, inhibit the up-regulation of hypoxia-responsive genes, whereas transition metals that can replace iron in the heme molecule can up-regulate these genes (Shih and Claffey, 1998).
**8.2.2 Non-oxygen dependent regulation**

Growth factors such as insulin-like growth factor-1, epidermal growth factor, basic fibroblast growth factor (Feldser et al., 1999; Elson et al., 2000) and hepatocyte growth factor (Tacchini et al., 2001) increase expression of HIF-1α, independent to hypoxia. HIF-1α, in turn, activates further insulin-like growth factors, therefore collaborating in the insulin-like growth factor loop found in various cancers, suggesting an important role of HIF-α1 in cellular energy metabolism (Feldser et al., 1999). HIF-1α also participates in the PI3 kinase/AKT pathway, shown to be important in cancer (Akakura et al., 2001; Semenza, 2000). Oncogenes, particularly v-SRC, have also been shown to induce HIF1α under normoxic conditions and superinduce HIF-1α during hypoxia with concomitant increase in HIF-1 up-regulated genes (Jiang et al., 1997).

**8.3 HIF-1 AND TUMOURIGENESIS**

Numerous studies in mice (Richard et al., 1999; Semenza et al., 2000) have demonstrated the importance of HIF-1α in normal growth and vascularisation. Mice deficient in HIF-1α showed abnormalities in embryogenesis and vascularisation, whereas mice heterozygous for HIF-1α developed normally yet displayed an impaired response to hypoxia (Semenza et al., 2000).

The role of HIF-1α in vascularisation during tumorigenesis appears to be more controversial.
HIF-1α depleted tumours, obtained by injecting HIF-1α negative embryonic stem cells into nude mice were less vascularised and less perfused than HIF-1α positive tumours, which displayed the characteristic tumour vascular pattern (Carmeliet et al., 1998). Yet, Ryan et al and Akakura et al have shown that loss of HIF-1α did not alter tumour vascularisation, therefore postulating that other pathways or factors must be implicated, such as elastase, which catalyses plasminogen into angiostatin, and found to be highly secreted by pancreatic cancer cells (Akakura et al., 2001; Ryan et al., 2000).

The effects of HIF-1α on tumour growth are also unclear.

Ryan et al found that HIF-1α is necessary for growth of a tumour in embryonic cell lines independent to its role in vascularisation (Ryan et al., 2000). In contrast, however, other studies have reported unimpaired growth and indeed better survival in the absence of HIF-1α (Blancher et al., 2000; Carmeliet et al., 1998). In breast cancer cell lines (MDA 435 and 231), (Blancher et al., 2000) cells showing best survival had least induced HIF proteins and high expression of VEGF. It was deduced that through non-hypoxia driven pathways (such as oncogenic activation of VEGF) a reduction of hypoxia-induced apoptosis and increased stress proliferation had occurred (Carmeliet et al., 1998).
9. HYPOXIA AND ANGIOGENESIS AS TARGETS FOR THERAPEUTIC STRATEGIES

9.1 Therapy to target hypoxia

It has been shown that hypoxic tumours correlate with poor prognosis. Therefore much interest is being generated in developing strategies to either target hypoxia directly, or use it as a means of delivery for novel chemotherapeutic regimes (Reviewed in Wouters et al., 2002).

Various stages in the hypoxia signalling pathway such as the HIF-1 activation pathway (vide supra) and genes regulated by hypoxia (glycolytic enzymes, glucose transporters, pro-angiogenic factors) may be targeted by specific therapy.

Bio-reductive drugs, activated by hypoxia, specifically target hypoxic tumour cells, thus facilitating tumour-specific chemotherapy.

Gene therapy using vectors directed to hypoxic areas is an expanding area of investigation. Anaerobic bacteria and macrophages - particularly in view of the evolving role of HIF-1 upregulation in macrophages (Burke et al., 2002) - have been postulated as possible vectors, and may act in combination with anti-angiogenic agents (Blancher and Harris, 1998).
9.2 Anti-angiogenic therapy

Since it has been established that angiogenesis plays a vital role in tumorigenesis, and many of the molecular mechanisms by which it does so have been elucidated, targeted therapies are being developed. Since the primary lesion is usually treated by surgery/irradiation (Ellis and Fidler, 1996; Gasparini, 1996), these agents are possibly useful as adjuvant therapy to stop the growth of micrometastases in patients. Halting or preventing growth is probably the main end-points of anti-angiogenesis therapy; tumour regression may not be achieved (Bicknell and Harris, 1996).

Anti-angiogenic agents are broadly classified as vascular growth factor inhibitors, protease inhibitors cytokine modulators and heparin-like agents (Bicknell and Harris, 1996). These agents interact with ECM molecules to disrupt cell migration and invasion (Gasparini, 1996), and down-regulate proliferating endothelial cells (therefore inhibiting vessel synthesis) at the site of neovascularisation, by up-regulating anti-angiogenic factors and targeting VEGF/VEGF receptors on microvessels (Gasparini, 1996). These agents, therefore, prevent further growth and aim to stabilise tumours (Ellis and Fidler, 1996) rather than contributing to tumour lysis (Folkman, 1995).

Anti-angiogenic agents are likely to work better alongside cytotoxic agents as chemotherapy targets cell components and anti-angiogenic therapy targets the endothelial components of tumours (Folkman, 1995; Gasparini, 1996).
Uptake of chemotherapy and radiotherapy may be increased as a result of anti-angiogenic therapy. As tumours grow, their vasculature becomes compressed encouraging areas of hypoxia and necrosis, thus tumour cells become tightly packed; angio-supression impeeds this process therefore facilitating the penetration of chemo and radio-therapeutic agents into tumour cells (Folkman, 1995). Angio-suppression also promotes apoptosis in tumour cells that may metastatic deposits dormant (Folkman, 1995; Gasparini, 1996).

Importantly, anti-angiogenic therapies are associated with low toxicity, as they are targeted to endothelial cells, and do not cross the blood brain barrier. Anti-angiogenic therapies develop little drug resistance, since endothelial cells are genetically stable and less likely to mutate into drug-resistant cells. In addition, they are not genotoxic so will not predispose to further malignancy (Bouck et al., 1996; Folkman, 1995; Gasparini, 1996). However, chronic long -term treatment (which produces optimum efficacy as evidenced by treatment of haemangiomas in children with interferon-alpha-2a) (Folkman, 1995) may produce low-grade resistant variants if single therapies are used, due to selection of less vascular dependent cells within heterogeneous tumours (Yu et al., 2001). Also, physiological angiogenesis would also be impaired (wound healing, bone repair, response to peripheral vascular disease) with potentially serious side effects (Ellis and Fidler, 1996), this therapy therefore needs to be targeted to avoid generalised angiosuppression (Gasparini, 1996).

More recently, a recombinant humanised anti-VEGF monoclonal antibody, bevacizumab, has been evaluated in randomised clinical trials in combination with 5FU and leucovorin for the treatment of metastatic colorectal cancer. These trials
have demonstrated that the combination of bevacizumab, 5FU and leucovorin significantly improved patient survival and response rates as compared with 5FU and leucovorin alone. These data, therefore, strongly support the use of anti-angiogenic agents in the management of metastatic colorectal cancer (Kabbinavar et al., 2003, Kabbinavar et al., 2005).

9.3 CAIX as a target for anticancer therapy

CAIX potentially provides a means of identifying hypoxia in tumours within the context of routine cancer biopsies, which would facilitate the identification of patients with poor prognosis for anti-hypoxia therapy (Loncaster et al., 2001).

Since CAIX has been shown to correlate with hypoxia and poor patient survival, (vide supra) and may have an important role in generating a suitable pH to confer cells with the ability to survive the effects of hypoxia, it may be an important, potential target for anti-tumour agents.

Acetazolamide, an aromatic sulphonamide, is a well-established, potent inhibitor of carbonic anhydrases and has more recently been investigated as an anti-tumour agent in combination with other cytotoxic agents. It has been shown that inhibition of membrane-bound Cas by 1, 3, 4-thiadiazole-2-sulfonamide derivatives inhibited tumour cells growth in a variety of cancer cell lines, possibly due to modifications in pH detrimental to tumour cells, such as diminished availability of bicarbonate for nucleotide synthesis (Supuran and Scozzafava, 2000; Supuran et al., 2001).
Specific enzyme inhibitors in the form of antibodies can provide a useful means to target CAIX in tumours. Radiolabelled mAbG250, a CAIX antibody, is being used in the detection of renal cancer in Europe and has demonstrated anti-tumour properties (Supuran and Scozzafava, 2000; Supuran et al., 2001).

9.4 EGFR as a target for anti-cancer therapy

Four potential mechanisms have been identified to target EGFR (reviewed by Seymour (Cox et al., 2000; Seymour, 2001) and Prenzel et al (Prenzel et al., 2001).

The principal two mechanisms are the use of chimerised human mouse antibodies such as cetuximab, and selective EGFR tyrosine kinase inhibitors such as ZD 1839 (Gefitinib; Iressa - Astrazeneca).

Tyrosine kinase inhibitors reduce activation of EGFR by binding to its ATP-binding sites. ZD1839 (gefitinib, or Iressa, Astra Zeneca) has been licensed in Japan and the United States of America as monotherapy following failure of standard chemotherapy for patients with NSCLC (Paez et al., 2004; Lynch et al., 2004). OSI-774 (erlotinib/tarceva from Roche, OSI pharmaceuticals and Genentech) is in phase II tirlas for NSCLC (Roskoski, 2004).

Monoclonal antibodies target the ligand- binding extracellular domain of the EGFR and block ligand-dependent receptor activation. Cetuximab (C225) is a mouse-human EGFR targeted antibody that modulates EGFR-potentiated signalling cascades and may potentiate the efficacy and overcome resistance to of established cytotoxic agents.
and to inhibit angiogenesis. Six clinical trials have shown very promising results and further studies are in progress (Reviewed by Starling and Cunningham, 2004).

Ligand-toxin complexes and anti-sense oligonucleotides are also being investigated. Neuregulin, EGF or TGFα are conjugated to bacterial exotoxins and following endocytosis, inhibit cellular protein synthesis resulting in cell death (Prenzel et al., 2001). Anti-sense oligonucleotides bind to EGFR mRNA and prevent specific protein transcription (Prenzel et al., 2001).

10. INTRODUCTION TO THE EXPERIMENTAL TECHNIQUES:
IMMUNOHISTOCHEMISTRY

10.1 An overview (Miller, 2002)

Immunohistochemistry is a technique utilised to identify constituents of a cell or tissue sample by using antibodies that bind to specific sites on these constituents which can then be visualised either by direct labelling of this so-called primary antibody, or by secondary labelling using a second antibody.

Antigens are cell constituents (proteins, carbohydrates or lipids) that display highly specific antibody binding sites. Tissue antigens are detected using specific, commercially available antibodies through immunohistochemical staining techniques.

Antibodies are proteins known as immunoglobulins, each consisting of a basic protein core structure formed by two light chains (kappa or lambda) and two heavy chains,
composed of constant and variable domains joined by inter- and intra-chain disulphide bonds. The variable domains distinguish the five different classes of immunoglobulins described, namely IgG, IgM, IgA, IgE and IgD, each with individual structural and antigenic properties.

IgG and IgM are the two principal antibodies used as anti-sera in immunohistochemistry. IgG is formed by two heavy chains and two light chains consisting of two monovalent antigen-binding fragments (Fab), two crystalline fragments (Fc) and one bivalent antigen binding fragment F(ab’)_2. Further structural specificity is achieved by hypervariable domains found on both light and heavy chains (forming part of the antigen combining site) and by the hinge region on the heavy chain (determined by different numbers of disulphide bonds). IgM consists of five subunits arranged as a pentamer, each composed of two heavy chains, two light chains and an additional J chain to increase stability.

Antibodies and antigens bind through the “lock and key” analogy: the antigen epitope and the variable domain side chains of the antibody are perfectly complementary and interlock, being held together by electrostatic forces, hydrogen bonds and van der Waals’ forces.

Polyclonal antibodies are immunohistochemically differing antibodies made by different cells, which bind to various epitopes on one specific antigen. They are primarily raised in rabbit (since rabbit serum rarely contains proteins recognisable by human antibodies and precipitates human proteins on a larger scale than serum from other ruminants) by injecting standard doses of the specified antigen. Blood is then
harvested from the rabbit and the antibodies isolated by various precipitation techniques.

Monoclonal antibodies are immunochemically identical clones of antibody made by plasma cells, which bind to a specific epitope on an antigen. These are primarily raised in mice. Following inoculation with the antigen, B lymphocytes are harvested as the antibody and a hybrid is created with mouse myeloma cells to increase antibody longevity. Non-reactive myeloma cells are then removed and the final antibody isolated. Monoclonal antibodies are homogenous thus decreasing the risk of non-specific antibody being present and inter-batch variability. However, cross reactivity is a potential hazard if the epitope to which the antibody is directed is present on other antigens.

The effectiveness of antibodies in immunohistochemistry relies on the titre and dilution utilised. Titre refers to the highest dilution of antiserum to stain a specific antigen with minimal background staining. Dilutions refer to the ratio between concentrated antibodies to volume of diluent, for example, a 1:10 dilution consists of one part concentrate to nine parts diluent giving a total of ten. Manufacturers normally recommend optimal antibody dilutions; however, investigators confirm the optimum dilution for a given tissue by dilution experiments.

In order for the antibody-antigen complex to be visualised by light microscopy, the complex needs to be labelled. Enzymes are the principle labelling molecules that, upon incubation with a chromogen, generates a certain colour. The most widely used
chromogen is 3, 3’-diaminobenzidine tetrahydrochloride (DAB). Horseradish peroxidase and alkaline phosphatase are the most common label enzymes. Both peroxidases and alkaline phosphatases are naturally found in most tissues. Background staining caused by hydrophobic/ionic protein-antibody interactions at other sites within the tissue section may also occur, recognised as a homogenous colour tinge. These endogenous substances and background staining must therefore be blocked to ensure that the end product is as a result of the antibody-antigen binding only. Hydrogen peroxide is commonly used to block endogenous peroxidases and levamisole blocks endogenous alkaline phosphatase. Incubating tissue with serum that will not interfere with the antigen-antibody complex reduces or eliminates background staining.

10.2 Immunohistochemical techniques (Miller, 2002)

Two main categories of immunohistochemical techniques are routinely employed, involving direct or indirect antibody-antigen links and visualisation. Direct techniques utilise enzyme linked primary antibodies directly onto the antigen followed by chromogen visualisation. Indirect techniques, which were the method of choice in this thesis, involve using an unconjugated primary antibody against the antigen, followed by a labelled secondary antibody against the primary antibody followed by chromogen. These methods are validated by substituting the primary antibody, which also serves to demonstrate the specificity of the antibody, and by using tissue controls (paraffin-embedded tissue sections known to either contain the antigen, in the case of positive controls, or not to contain the antigen in the case of negative controls).
11. SUMMARY OF EXPERIMENTAL WORK RELATING TO THIS SERIES OF RESECTED COLORECTAL LIVER METASTASES

The current series of resected colorectal liver metastases was used by CD Sutton MD, FRCS, to evaluate the expression and prognostic significance of angiogenesis as measured by the immunohistochemical detection of microvessel density, vascular endothelial growth factor, thrombospondin, p53 and thymidine phosphorylase.

The most important findings were:

1) High tumour edge hotspot microvessel density was an independent negative prognostic factor. The tumour edge is the most biologically active area of the tumour, therefore understandably more vascular, and thus correlating with poor survival.

2) Absent stromal VEGF was an independent negative prognostic factor. Stromal tumour associated macrophages expressing VEGF may be tumoricidal.
12. AIMS OF THE STUDY

Hypoxia, angiogenesis and deregulated cellular proliferation have been shown in numerous studies to promote tumorigenesis and affect patient survival. We have already shown the prognostic relevance of VEGF and microvessel density in colorectal liver metastases.

In order to further enhance the understanding of the behaviour of colorectal liver metastases, and potential prognostic implications, the overall aim of this thesis was to evaluate the hitherto undescribed inter-relationships and prognostic significance of hypoxia, angiogenesis, and extracellular matrix remodelling, as determined by the expression of CAIX, HIF-1α, MVD, EGFR, MMP-2 and MMP-9 in a series of resected colorectal liver metastases.

The following objectives were therefore established:

1) To determine the protein expression by immunohistochemical techniques of CAIX, HIF-1α, EGFR, MMP-2 and MMP-9 in a series of resected colorectal liver metastases.

2) To correlate the protein expression of the above factors with MVD (already established in this series).

3) To determine the prognostic significance of these factors with regards to patient survival.

4) To propose a theory of tumour behaviour which may not only enhance our understanding of tumour biology, but in the future may facilitate tumour-specific chemotherapeutic treatment strategies.
METHODS
1. THE STUDY: PATIENTS AND ETHICS

1.1 Study population

All patients who underwent a liver resection for colorectal liver metastases from 1993-1999 at the University Hospitals of Leicester NHS Trust and the Royal Liverpool Hospital were identified. Data were collected prospectively in Liverpool. In Leicester, data were collected retrospectively until 1999 and prospectively thereafter. In Leicester, a database was constructed by reviewing patients’ case notes detailing patient demographics, location of the primary tumour, histological characteristics of the primary tumour, pre-operative chemotherapy and radiotherapy, surgical management of the primary tumour, post-operative chemotherapy, temporal relationship of the diagnosis of liver metastases in relation to the primary tumour (synchronous or metachronous), number, position and size of the liver metastases, surgical management of the liver metastases including post-operative morbidity/mortality, site and extent of tumour recurrence, management of recurrence and survival data. The Royal Liverpool Hospital prospective database was obtained in anonymised form (in agreement with the Caldicott code of practice) from the Experimental Officer at the Tissue Bank.

Patient survival was measured from the date of hepatic resection to the patient’s date of death or to the current date if the patient was still alive at the time of writing.
To establish whether a patient was dead or alive, three principle sources were used:

1) The Hospital Information and Services System and the Apex Histopathology database (University Hospitals of Leicester), which record patient details and should document whether a patient has died.

2) The patient’s General Practitioner as obtained from the hospital records.

3) Cancer Registries.

Paraffin-embedded tissue blocks pertaining to each resected liver metastasis were used. Suitable tumour edge biopsy sections were identified by reviewing Haematoxylin and Eosin sections of each tumour block, in conjunction with a Consultant Histopathologist.

1.2 Ethics and Consent

This project was submitted for approval to the Leicestershire Research Ethics Committee, the Directorate of Research and Development representing the University Hospitals of Leicester NHS Trust, the Liverpool Research Ethics Committee, and the Research and Development Office representing the Royal Liverpool Hospital.

In Liverpool, patients were prospectively and specifically consented for surplus tissue to be stored in the Tissue Bank, and for its subsequent use in biomedical research.
In Leicester, patients had not been specifically consented for the storage and further use of surplus tissue, and a Tissue Bank had not been established. No guidelines were in place for the use of archival material. Following the highly publicised Alder Hey Children’s Hospital and Bristol Royal Infirmary enquiries, much local debate ensued regarding the issue of retained tissue and patient consent, all at the time of project submission. The following paragraphs summarise these issues and eventual local policy with regards to the use of archival material for research purposes.

In 1995, the Nuffield Council on Bioethics published a document entitled “Human Tissue, Ethical and Legal Issues” (www.nuffieldfoundation.org/bioethics) which in essence disregarded the need to obtain specific consent for the use of surplus material for research purposes, on the basis of the following criteria:

1) Once adequate consent for a procedure is obtained, subsequent uses of the tissue by implication have been agreed to, and the principle of abandonment is applied, provided that appropriate ethical, legal and professional codes of conduct have been followed (13.12). This is derived in part from the legal stance that tissue is not property, thus a person retains no claim to tissue once it has been removed, and from the Human Tissue Act 1961, the Human Organ Transplants Act 1989 and the Anatomy Act of 1984 which were interpreted as promoting the principle that once tissue was removed, it was done so “free of all claim” (13.26). The use of such surplus material for research should be approved by a local ethics committee (13.38; Appendix 3, paragraph 3), and obtained from organised storage through tissue banks and registers (13.31-13.33).
2) A utilitarian approach to research on surplus material. The Council advocated the importance of using archival tissue removed during a medical procedure for the benefit of the population and patients as a whole rather than the benefit to one patient of retaining such tissue due to personal wish (Appendix 2, paragraph 7).

However, in the advent of the Bristol Royal Infirmary (www.bristol-inquiry.org.uk) and the Alder Hey Children’s Hospital in Liverpool inquiries (www.rlciquiry.org.uk), fundamental changes to the entire concept of organ and tissue retention and patient consent has ensued.

In 2001, the Chief Medical Officer, in a document entitled “The Removal, Retention and Use of Human Organs and Tissue from Post Mortem Examination” (www.doh.gov.uk) highlighted the need for changes to the law with regards to these practices, followed by the consultation document “Human Body, Human Choices” in 2002 (www.doh.gov.uk) and an interim statement of the “Use of Human Organs and Tissues” in April 2003 (www.doh.gov.uk). These reports addressed the need to change the hitherto practices of organ and tissue use, by advocating patient consent as the framework upon which new legislation should be based. The former principles derived from the Human Tissue Act 1961, the Human Organ Transplants Act 1989 and the Anatomy Act of 1984, as discussed above, were not deemed to be in keeping with changes in social attitudes and expectations experienced in today’s society. The responses to “Human Body, Human Choices” derived from a wide field of practitioners were generally in favour of specific consent for the storage and subsequent use of tissue specimens, such as for research.
It is evident that patients therefore need to be specifically consented for the storage and use of surplus tissue in ethically approved research, using appropriately directed information leaflets. This clearly encompasses prospective consent. However, the use of archival material is still under consideration by the Department of Health. “The use of human organs and tissue: an interim statement” published in April 2003 by the Clinical Ethics and Human Tissue Branch of the Department of Health (www.doh.gov.uk) stipulates “where any new use of stored tissue is proposed, a decision will need to be taken as to whether consent (or further consent) needs to be sought.” The document subsequently advises that if consent was given for research in the first instance, and is deemed to be valid today, research may continue. If however, identifiable patients have not given consent, “consideration should be given to whether it is possible (or, depending on the nature of the research, necessary) to seek consent”. The decision to seek consent would therefore, at present, appear to be the responsibility of local ethics committees and the appropriate colleges, until legislation is clarified.

The Medical Research Council, in “Human Tissue and Biological Samples for use in Research” (www.mrc.ac.uk) firmly states that prospective consent must be acquired to use surplus tissue for research. However with regards to archival material it states that, with ethics committee approval “old samples of material surplus to clinical requirements may be used for linked research without specific consent if there is no possibility that the research could affect the patient’s interests in any way and if obtaining individual consent is not practicable”. These guidelines have been supported by the Royal College of Pathologists in 2001 (www.rcpath.org).
Further to considering consent for the use of human tissue for research purposes, these institutions have also emphasized the need to gain patient consent for the use of information contained in the medical case notes, in accordance with the Data Protection Act of 1998. Specifically, the confidentiality and security of patient data held in either manual or electronic form must be maintained in accordance with the Caldicott code of practice (www.croydon.gov.uk/Caldicott/Page5.htm).

It can therefore be surmised that consent for the use of archival material has not been legislated upon to date, does not appear to be a definitive requisition either by the Royal College of Pathologists or the Medical Research Council, and at this present time will be determined by local trusts and ethics committees.

It was the view of the Directorate of Research and Development, acting for the Trust, and the Leicestershire Research Ethics Committee that retrospective consent should be sought.

Upon discussion with the Business Manager of the Directorate of Research and Development, the investigator and the Supervising Consultant, the following criteria were defined:

1) Deceased patients were not approached, which by implication suggested that their relatives were not contacted.

2) Patients alive at the time of research and under follow-up in the Surgical Outpatient clinic were approached for consent. This entailed sending an
information leaflet and a consent form, asking for a reply within two weeks. If no reply was obtained, a further letter was sent and if no reply was obtained once again, no further action was taken and the specimens used.

3) If it was unclear from the hospital records as to whether the patient was alive or dead, the General Practitioner was contacted, asking him/her to indicate whether the named patient was dead or alive, and if alive, if he/she, in their opinion should be approached for consent to use the stored tissue. If the General Practitioner confirmed the patient to be alive and well to be approached for consent, this was undertaken as detailed above. If the patient had died, or moved away to an address unknown to the General Practitioner, no further action was taken and the samples used.

Information leaflets, detailing issues of tissue storage and its potential use in research, and consent forms were agreed.

2. IMMUNOHISTOCHEMICAL DETECTION KITS (DAKO ™ Ely, UK)

2.1 The Dextran Polymer Conjugate Two-step Visualisation system, Envision

This kit is a two-stage visualisation system that uses dextran polymers conjugated to the secondary antibody followed by chromogen:

1) The primary mouse antibody is applied to the tissue section to bind to the antigen.

2) The secondary goat anti-mouse immunoglobulin conjugated to a peroxidase-labelled polymer binds to the primary antibody.
3) Substrate-chromogen solution (DAB – see Table 2.2) reacts with the peroxidase to generate a brown precipitate visualised by light microscopy.

2.2 ChemMate system

This method consists of a labelled streptavidin biotin visualisation system:

1) The primary mouse or rabbit antibody is applied to each slide
2) Horseradish peroxidase conjugated streptavidin binds to the primary antibody
3) A biotin-labelled secondary antibody detects several peroxidase conjugated streptavidin molecules.
4) The substrate chromogen solution reacts with the peroxidase to form the brown precipitate, as above.

2.3 The avidin biotin alkaline phosphatase (ABC) method

This method uses the affinity of streptavidin (avidin) for biotin:

1) The primary mouse antibody is applied to the tissue section to bind to the antigen.
2) A secondary anti-mouse, biotin-bound antibody binds to the primary antibody.
3) The pre-formed (Strept) Avidin-Biotin-enzyme Complex (ABC) containing biotinylated alkaline phosphatase binds to the biotin on the secondary antibody.
4) A substrate-chromogen solution (NBT/BCIP – see Table 2.4) detects the alkaline phosphatase via a redox reaction: BCIP oxidises AP producing an indigo precipitate, which is further reduced by NBT to generate the final intense indigo precipitate that is visualised by light microscopy.

2.4 The Catalysed Signal Amplification (CSA) system

This system uses the streptavidin-biotin-peroxidase complex:

1) The primary mouse antibody is applied to the tissue section.
2) A biotinylated secondary antibody is used to detect the primary antibody.
3) The streptavidin in the streptavidin–biotin-peroxidase complex detects this secondary antibody by binding to its biotin.
4) A biotin phenol precipitate is formed, catalysed by the peroxidase on the streptavidin–biotin-peroxidase, which amplifies the number of biotin molecules available to bind to the streptavidin–biotin-peroxidase.
5) Visualisation is then achieved with substrate chromogen solution.

2.5 The Catalysed Signal Amplification (CSA) Ancillary System

Intended for use in conjunction with the CSA System, the CSA Ancillary system is recommended to reduce non-specific background staining without affecting the activity of the primary antibody. It comprises a concentrated buffer solution, antibody diluent and a biotin blocking system (containing avidin and biotin).
3. MATERIALS AND METHODS: THE EXPERIMENTAL TECHNIQUES

The following paragraphs summarise the overall techniques utilised for protein immunodetection. A summary is presented herein (Tables 2.1-2.4).

3.1 Slide preparation

Formalin-fixed, paraffin-embedded dehydrated tissue blocks were cut into 5μm sections using a microtome, and mounted on vectabond (Vector Laboratories, UK) or silane-coated slides (Surgipath, UK) to aid tissue adhesion. The slides were stored in a cold room at 4°C to preserve antigenicity.

3.2 Buffers

Tris-buffered saline was utilised to wash unbound antibody from slides before incubation of the next antibody to prevent antibody-antigen complexes precipitating onto the section (Miller, 2002). For details of how buffers were prepared, see “Standard Operating Procedures, section 8”.

3.3 Antigen retrieval

The formalin-based fixation process and the embedment of tissue in paraffin wax may hide some antigen epitopes, firstly through the denaturation of the protein structure by paraffin and secondly through cross-linking by fixation in formaldehyde, with
concurrent loss of antigenicity. These epitopes therefore need unmasking before immunohistochemical techniques can be employed (Miller, 2002).

Epitope retrieval can be undertaken by proteolytic pre-treatment, heat-based pre-treatment and the use of target retrieval buffers, often recommended by the manufacturer of the antibody, with the aim of restoring protein structure, and destroying the cross-links (DAKO, 2000).

Proteolytic pre-treatment involves the use of enzymes for antigen retrieval, principally, trypsin, pepsin, pronase and proteinase K, optimised in accordance with the fixation procedures and antibody being used by each laboratory. Heat-based pre-treatments involve the optimised use of microwave oven heating, pressure-cooking, water bath heating autoclaving and steamer heating (DAKO, 2000). A more detailed account of the techniques relevant to this study is described in the Section entitled “Antigen Retrieval Techniques, section 8.4”.

3.4 Statistical analysis

Statistical analysis and graphic presentation was performed using SPSS software for Windows version 11. The Chi-squared test was employed to test for relationships between categorical tumour variables. Survival curves were plotted using the Kaplan-Meier method, and the log-rank test was used to determine statistical differences between life tables. A Cox proportional hazard model was used to assess the effects of patient and tumour variables on overall survival and identify any independent prognostic factors. A p-value = 0.05 was considered significant.
**Table 2.1 Summary of immunohistochemical visualisation methods**

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Primary Antibody</th>
<th>Dilution of Antibody</th>
<th>Incubation</th>
<th>Diluent</th>
<th>Secondary Antibody</th>
<th>Positive control</th>
<th>Antigen retrieval</th>
<th>Endogenous peroxidase block</th>
<th>Background staining block</th>
<th>Method</th>
<th>Visualisation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbonic anhydrase IX</td>
<td>M75 monoclonal antibody</td>
<td>1:50</td>
<td>30 minutes at room temperature</td>
<td>5% normal human serum in TBS</td>
<td>Peroxidase labelled polymer conjugated to goat anti-mouse immunoglobulin (EnVision, DAKO) for 30 minutes</td>
<td>NSCLC Bile ducts internal control</td>
<td>Non required</td>
<td>0.5% H₂O₂</td>
<td>10% normal human serum in TBS for 15 minutes</td>
<td>EnVision (Dako)</td>
<td>DAB (Envision kit, DAKO) for 5 minutes Meyer’s haematoxylin for 15 seconds</td>
</tr>
<tr>
<td>EGFR</td>
<td>EGFR 113 monoclonal antibody</td>
<td>1:20</td>
<td>60 minutes at room temperature</td>
<td>5% normal rabbit serum in TBS</td>
<td>Peroxidase labelled polymer conjugated to goat anti-mouse immunoglobulin (EnVision, DAKO) for 30 minutes</td>
<td>NSCLC Bile ducts internal control</td>
<td>Pressure cook in citrate buffer for 2 minutes</td>
<td>0.5% H₂O₂</td>
<td>1:5 normal rabbit serum in TBS for 10 minutes</td>
<td>EnVision (Dako)</td>
<td>DAB (Envision kit, DAKO) for 5 minutes</td>
</tr>
<tr>
<td>HIF-1α</td>
<td>H1667 monoclonal antibody</td>
<td>1:1000</td>
<td>Overnight at 4°C</td>
<td>Antibody diluent with background reducing components (CSA Ancillary System, DAKO)</td>
<td>Biotinylated rabbit antimouse (CSA, DAKO) for 15 minutes</td>
<td>Kidney Renal cell carcinoma Bile ducts internal control</td>
<td>97° Water bath in antigen retrieval solution (DAKO)</td>
<td>6% H₂O₂</td>
<td>Biotin blocking system (CSA Ancillary system) serum-free protein in PBS (CSA)</td>
<td>CSA streptavidin-biotin-peroxidase (DAKO) CSA Ancillary System</td>
<td>DAB for 5 minutes (CSA kit, DAKO) Meyer’s haematoxylin for 15 seconds</td>
</tr>
<tr>
<td>MMP-2</td>
<td>Anti-MMP-2 mouse monoclonal</td>
<td>1:50</td>
<td>30 minutes at room temperature</td>
<td>5% normal rabbit serum in TBS</td>
<td>Peroxidase labelled polymer conjugated to goat anti-mouse immunoglobulin (EnVision, DAKO) for 30 minutes</td>
<td>NSCLC Bile ducts internal control</td>
<td>Pressure cooking in citrate buffer for 2 minutes</td>
<td>0.5% H₂O₂</td>
<td>None</td>
<td>EnVision (Dako)</td>
<td>DAB (Envision kit, DAKO) for 5 minutes Meyer’s haematoxylin for 15 seconds</td>
</tr>
<tr>
<td>MMP-9</td>
<td>Anti-MMP-9 mouse monoclonal</td>
<td>1:100</td>
<td>30 minutes at room temperature</td>
<td>5% normal rabbit serum in TBS</td>
<td>Peroxidase labelled polymer conjugated to goat anti-mouse immunoglobulin (EnVision, DAKO) for 30 minutes</td>
<td>NSCLC Bile ducts internal control</td>
<td>Pressure cooking in citrate buffer for 2 minutes</td>
<td>0.5% H₂O₂</td>
<td>None</td>
<td>EnVision (Dako)</td>
<td>DAB (Envision kit, DAKO) for 5 minutes Meyer’s haematoxylin for 15 seconds</td>
</tr>
</tbody>
</table>
Table 2.2 Reagents contained in the immunohistochemistry detection systems

<table>
<thead>
<tr>
<th>KIT</th>
<th>BOTTLE</th>
<th>REAGENT</th>
<th>COMPOSITION</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABC/AP*1</td>
<td></td>
<td>Reagent A</td>
<td>Streptavidin in 0.01mMol/L phosphate buffer, 0.15 sodium chloride, 15 mMol/L NaN₃ at pH 7.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reagent B</td>
<td>Biotinylated alkaline phosphatase in 0.05 mmol/L Tris/HCl, 0.1mol/L sodium chloride, 1mmol/L magnesium chloride, 0.1mmol/L zinc chloride, 15mmol/L NaN₃ at pH 7.2</td>
</tr>
<tr>
<td>Envision*1</td>
<td>1</td>
<td>Peroxidase block</td>
<td>0.03% hydrogen peroxide with sodium azide</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Labelled polymer</td>
<td>Peroxidase labelled polymer conjugated to goat anti-mouse immunoglobulins in Tris/HCl buffer with carrier protein and anti-microbial agents</td>
</tr>
<tr>
<td></td>
<td>3a</td>
<td>Buffered substrate solution</td>
<td>pH 7.5 with hydrogen peroxide and a preservative.</td>
</tr>
<tr>
<td></td>
<td>3b</td>
<td>DAB + chromogen</td>
<td>3,3'-diaminobenzidine chromogen solution</td>
</tr>
<tr>
<td>CSA*1</td>
<td>1</td>
<td>Peroxidase block</td>
<td>3% hydrogen peroxide in water</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Protein block</td>
<td>Serum free protein in phosphate buffered saline with 0.015M sodium azide</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Link antibody</td>
<td>Biotinylated rabbit anti-mouse with 0.015M sodium azide in Tris/HCl with carrier protein</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>Streptavidin-biotin complex reagent A</td>
<td>Streptavidin in phosphate buffered saline (PBS)</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>Streptavidin-biotin complex reagent B</td>
<td>Biotin conjugated to horseradish peroxidase in PBS</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>Streptavidin-biotin complex Diluent</td>
<td>PBS buffer with carrier protein</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>Amplification reagent</td>
<td>Biotinylated tyramide with hydrogen peroxide in PBS</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>Streptavidin-peroxidase</td>
<td>Streptavidin conjugated to horseradish peroxidase in PBS</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>DAB substrate chromogen tablets</td>
<td>10mg 3-3' daminobenzidine tetrahydrochloride with dessicant</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>Substrate</td>
<td>Tris buffer concentrate</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>Substrate peroxide</td>
<td>0.8% in water</td>
</tr>
<tr>
<td>CSA Ancillary System*1</td>
<td></td>
<td>Biotin blocking system</td>
<td>15mL avidin</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Biotin blocking system</td>
<td>15mL biotin</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TBST</td>
<td>500mL 10x concentrate of tris buffered saline with Tween 20</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Antibody diluent</td>
<td>Diluent for use in the preparation of primary antibodies to reduce background staining</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Target retrieval solution</td>
<td></td>
</tr>
<tr>
<td>ChemMate*</td>
<td>A</td>
<td>Link, biotinylated secondary antibody</td>
<td>Biotinylated goat anti-mouse and anti-rabbit Ig in buffer solution</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>Streptavidin peroxidase</td>
<td>Streptavidin conjugated to horseradish peroxidase in buffer solution</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>DAB</td>
<td>3-3' diaminobenzidine tetrahydrochloride in organic solvent</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>HRP substrate buffer</td>
<td>Buffered solution containing hydrogen peroxide</td>
</tr>
</tbody>
</table>
Table 2.3 Summary of primary antibodies for immunohistochemistry

<table>
<thead>
<tr>
<th>ANTIBODY</th>
<th>ANTIGEN</th>
<th>SUPPLIER</th>
<th>DESCRIPTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>M75</td>
<td>N-terminal domain of MN/CAIX</td>
<td>Gift from Prof A Harris, Oxford</td>
<td>Murine monoclonal antibody</td>
</tr>
<tr>
<td>EGFR 113 NCL</td>
<td>Extracellular domain of the</td>
<td>Novocastra Laboratories Limited</td>
<td>Mouse monoclonal IgG2a</td>
</tr>
<tr>
<td></td>
<td>epidermal growth factor receptor</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HIF-1 alpha antibody</td>
<td>HIF-1 alpha</td>
<td>Abcam Limited, Cambridge, UK</td>
<td>Mouse monoclonal IgG2b fusion protein derived from amino acids 432-528 of human HIF-1 alpha</td>
</tr>
<tr>
<td>(ab463)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-MMP-2 clone 56-</td>
<td>Residue 468-483 of latent and active</td>
<td>Chemicon International Ltd</td>
<td>Mouse monoclonal IgG1/k</td>
</tr>
<tr>
<td>5D11</td>
<td>MMP-2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti MMP-9 clone 56-</td>
<td>Residues 626-644 of the carboxyl</td>
<td>Chemicon International Ltd</td>
<td>Mouse monoclonal IgG1/k</td>
</tr>
<tr>
<td>2A4</td>
<td>terminal of active and latent MMP-9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-TIMP-2 clone 67-</td>
<td>Carboxyl terminal of TIMP-2</td>
<td>Chemicon International Ltd</td>
<td>Mouse monoclonal IgG1/k</td>
</tr>
<tr>
<td>4H11</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2.4 Summary of general reagents

<table>
<thead>
<tr>
<th>REAGENT</th>
<th>SUPPLIER</th>
<th>COMPOSITION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris buffered saline (TBS)</td>
<td>Roche Diagnostics, USA</td>
<td>0.05M Tris/HCl, 0.15M NaCl at pH 7.5</td>
</tr>
<tr>
<td>Citrate acid buffer</td>
<td>AnalR</td>
<td>10mM citric acid at pH 6.0</td>
</tr>
<tr>
<td>NBT/BCIP tablets for</td>
<td>Roche Diagnostics, USA</td>
<td>0.4 mg/ml Nitroblue tetrazolium chloride (NBT)</td>
</tr>
<tr>
<td>visualisation solution</td>
<td></td>
<td>0.19mg/ml 5 bromo 4 chloro 3 indolyl phosphate toluidine salt (BCIP)</td>
</tr>
<tr>
<td>For use with ABC/AP system</td>
<td></td>
<td>100mM Tris buffer at pH 9.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50mM MgSO4</td>
</tr>
<tr>
<td>Levamisole (for visualisation</td>
<td>Gift, Specials Histopathology, Dept of</td>
<td>1M levamisole to block endogenous alkaline phosphatase</td>
</tr>
<tr>
<td>solution)</td>
<td>Pathology, LRI</td>
<td></td>
</tr>
<tr>
<td>Mayer’s haematoxylin</td>
<td>Reconstituted by the Cancer Translational</td>
<td>1mM sodium iodate, 303mM citric acid, 105mM aluminium potassium sulphate, 5Mm citric acid</td>
</tr>
<tr>
<td></td>
<td>research Group, Glenfield General Hospital</td>
<td></td>
</tr>
</tbody>
</table>
4. METHODS FOR CARBONIC ANHYDRASE IX

4.1 Immunohistochemical detection kit

The Dextran Polymer Conjugate Two-step Visualisation system, Envision, was used.

4.2 Immunohistochemical method

2 litres of TBS buffer were prepared (see Standard Operating Procedure 8.1).
Racked slides were placed in a 60° oven for 5 minutes to melt the embedding wax.

Twenty-five racked slides were dewaxed in xylene (Genta Medical) and re-hydrated
by periodic agitation for 2 minutes through a series of alcohol solvents (99% and 95%
industrial methylated spirit, Genta Medical) under a fume hood, and washed in cold
running tap water.

The racked slides were placed in an appropriately sized plastic container and bathed
in TBS for 5 minutes on a magnetic stirrer. Upon completion of the wash, excess
buffer was wiped away from the corners of each slide with soft tissue paper and then
aligned in a humid chamber, to prevent the sections drying out.

Endogenous peroxidase was blocked for 30 minutes using 2 – 3 drops of 0.5%
hydrogen peroxide (sufficient to cover the entire specimen on the slide) from the
Envision kit, followed by 5 minutes buffer wash.
The slides were once again placed in the humid chamber and 100 µl of 5% normal human serum was applied using a calibrated Gilson’s pipette for 15 minutes to block background staining. The slides were racked and washed in fresh buffer.

100 µl of primary antibody was applied using a calibrated Gilson’s pipette onto each slide and incubated for 30 minutes in the humid chamber at room temperature.

Primary antibody was substituted by TBS for the negative control. The slides were washed in buffer and two drops of secondary polymer (Bottle 2) were applied to each slide for 30 minutes. Bathing in TBS followed for 5 minutes.

The substrate-chromogen solution was prepared by mixing 1ml of buffered substrate solution (Bottle 3a) and 1 drop of 3,3' diaminobenzidine chromogen solution, DAB (Bottle 3b) per 10 slides as supplied in the kit. 3 drops of substrate-chromogen solution were applied per slide, using the plastic Pasteur pipette supplied, and incubated for 5 minutes.

The slides were washed in cold running tap water for 5 minutes and counterstained with Mayer’s Haematoxylin for 15 seconds, placed in running water until the water ran clear and dehydrated using graded alcohol solvents. Cover slips were mounted in a fume cupboard by applying one drop of DPX (BDH Chemicals, England) obtained by immersing the tip of an orange stick into the flask containing the solution, onto a cover slip and laying the slide, tissue side down onto the cover slip. The loosely adhered cover slip was then fixed into position with gentle pressure using another orange stick. The slides were allowed to dry.
4.3 Method optimisation

To ascertain the correct dilution of M75 to enable good quality, interpretable staining different antibody dilutions were utilised in the method above.

4.4 Assessment of immunohistochemistry for CAIX

A semi-quantitative grading scale was used to assess the quantity and distribution of CAIX expression in each slide. Slides were scanned by light microscopy (LJM and 50% independently by a Consultant Histopathologist) at low power (x40 and x100) to determine the overall percentage of positively staining tumour cells, stromal staining and tumour necrosis. High power field was used (x200) to examine the percentage membranous and cytoplasmic protein expression of CAIX. Median percentage membranous and cytoplasmic staining values were calculated and used for data analysis.

Tumour necrosis (TN) was assessed using the following grading scale: 0 = no necrosis; 1 = minimal necrosis: focal areas of TN affecting < 25% of the tumour; 2 = moderate necrosis: widespread areas of TN affecting 25 – 75% of the tumour; 3 = extensive necrosis: TN involving >75% of the tumour (Leek et al., 1999; Swinson et al., 2003). Tumours with ≥ median tumour necrosis were considered extensively necrotic; those with<median tumour necrosis were considered focally necrotic.
5. METHODS FOR EGFR

5.1 Immunohistochemical detection kit

The Dextran Polymer Conjugate Two-step Visualisation system, Envision was used.

5.2 Immunohistochemical method

2L TBS were prepared (Standard Operating Procedure 8.1). Twenty-five racked slides were dewaxed in xylene and rehydrated by periodic agitation through graded alcohol solvents (99% and 95%) and washed in cold running tap water. The racked slides were placed in an appropriately sized plastic container and bathed in TBS for 5 minutes on a magnetic stirrer. Upon completion of the wash, excess buffer was wiped away from the corners of each slide, which were then placed in a humid chamber for incubation.

Antigen retrieval by pressure-cooking was undertaken (See SOP 8.4.1). Endogenous peroxidase was blocked for 5 minutes by applying two-three drops of 0.5% hydrogen peroxide (Bottle 1), followed by 5 minutes buffer wash. 100 μl of normal rabbit serum, diluted to a 1:20 concentration in TBS was pipetted onto each slide for 15 minutes, to block background staining block. Buffer wash followed.

100 μl of EGFR.113 NCL primary antibody (Novocastra, UK) was applied using a calibrated Gilson’s pipette onto each slide and incubated for 60 minutes at room temperature. Primary antibody was substituted by TBS for the negative control. The slides were washed in buffer and two drops of secondary polymer (Bottle 2) were
applied to each slide in the humid chamber for 30 minutes. Bathing in TBS followed for 5 minutes.

The substrate-chromogen solution was prepared by mixing 1ml of buffered substrate solution (Bottle 3a) and 1 drop of DAB+chromogen (Bottle 3b) per 10 slides as supplied in the kit. Three drops of substrate-chromogen solution were applied per slide and incubated in the humid chamber for 5 minutes. The slides were washed in cold running water for 2 minutes, counterstained with Mayer’s haematoxylin for 15 seconds, and washed again thoroughly in cold running water until it ran clear. The slides were dehydrated using graded alcohol solvents, and cover slips were mounted using DPX (BDH Chemicals, England).

4.3 Method optimisation: Immunohistochemical staining methods

During optimisation, the ChemMate system (DAKO, Ely, UK) was utilised first due to its successful results in bladder cancer specimens. The avidin biotin alkaline phosphatase (ABC) method (DAKO, Ely, UK) was tried next, due to its successful use in breast cancer specimens at the Breast Cancer Research Unit, Glenfield General Hospital.

4.3.1 ChemMate method

Dewaxed and rehydrated vectabond coated-slides, as detailed above, were rinsed in distilled water. Antigen retrieval was performed using pressure-cooking in citrate
buffer, as described in Standard Operating Procedures 8.4.1. The racked slides were washed in TBS for 2 minutes and aligned in the humid chamber.

100μls of EGFR.113 NCL primary antibody (Novocastra, UK) diluted in normal rabbit serum at a 1:20 concentration was applied and incubated for 1 hour at room temperature, followed by two 5 minutes washes in TBS. Primary antibody was substituted by TBS for the negative control. Two or three drops (as required to ensure full specimen coverage) of ChemMate hydrogen peroxide block were applied to each section for 5 minutes followed by wash in buffer. This process was repeated. One drop of ChemMate secondary complex (Bottle A) was applied to each section and incubated for 25 minutes at room temperature. Two five minute washes in TBS followed. One drop of ChemMate tertiary complex (Bottle B) was applied to each section for 25 minutes at room temperature, followed by two five minute TBS washes.

The substrate-chromogen solution was prepared as follows:

**Table 2.5 Substrate-chromogen preparation using the ChemMate system**

<table>
<thead>
<tr>
<th></th>
<th>For 1 slide</th>
<th>5 slides</th>
<th>10 slides</th>
<th>50 slides</th>
</tr>
</thead>
<tbody>
<tr>
<td>HRP substrate buffer Bottle D</td>
<td>750μl</td>
<td>4ml</td>
<td>8ml</td>
<td>40ml</td>
</tr>
<tr>
<td>DAB solution</td>
<td>15μl</td>
<td>80μl</td>
<td>160μl</td>
<td>800μl</td>
</tr>
</tbody>
</table>

100μls of solution were applied to each slide in the humid chamber and incubated for 10 minutes. The solution was washed-off in cold running tap water. The slides were counterstained with Meyer’s haematoxylin for 15 seconds and thoroughly washed in cold running tap water until the latter ran clear. Cover slips of an appropriate size were mounted using DPX (BDH Chemicals, England) as already described.
Optimisation

The positive control slide, liver sections and colon sections were incubated with different antibody concentrations and a corresponding negative control.

4.3.2 The avidin-biotin complex alkaline phosphatase method

Dewaxed and rehydrated vectabond coated-slides, as detailed above, were rinsed in distilled water.

Antigen retrieval by pressure-cooking was performed (SOP 8.4.1). Following antigen retrieval, the racked slides were transferred into a jar containing approximately 500mls of 2% hydrogen peroxide in distilled water to block endogenous peroxidase for 30 minutes.

100μls normal rabbit serum was applied to each section using a Gilson’s pipette for 10 minutes to block background staining. The excess serum was gently blotted away from the slide, not washed.

100μls of EGFR.113 NCL (primary antibody) diluted in normal rabbit serum at a 1:20 concentration was applied and incubated overnight at 4°C. Primary antibody was substituted by TBS for the negative control.

The following day, the ABC solution was prepared as follows, and allowed to stand for 30 minutes to enable the complex to form:

1000μl TBS
1µl Streptavidin (Reagent A)
1µl Biotinylated alkaline phosphatase (Reagent B)

Slides were placed in the rack, washed in TBS buffer for 5 minutes and aligned in the humid chamber. 100µls of biotinylated rabbit anti-mouse secondary antibody was pipetted onto each slide and incubated for 30 minutes at room temperature. TBS wash followed. 100µls of the ABC solution was incubated on each slide for 30 minutes.

The NBT/BCIP development solution was prepared by dissolving one NBT/BCIP tablet (Roche, France) in 10ml distilled water and applied to each slide. Subsequently, 1µl of levamisole was added, in order to block endogenous alkaline phosphatase in the liver sections. The positive control slide and one liver section slide were checked by light microscopy at 10 minutes intervals until clear tissue staining had been achieved. The slides were washed in buffer, rinsed in cold running tap water followed by de-ionised water. The slides were not rehydrated through graded alcohols and xylene, as this process dissolves the staining pattern obtained. Aqueous mount (see below) was used to apply appropriate cover slips.

**Optimisation**

1) Since this method had been used with success in breast cancer at the Breast Cancer Research Unit, Glenfield General Hospital, one of their breast cancer sections was utilised (Courtesy of Mrs Karen Kulblicki) as a positive control. Different antibody concentrations were incubated on liver and colon sections, in addition to the positive control.
2) The substrate-chromogen solution was prepared in two different ways. At first, 600μl NBT and 400μl BCIP were used to yield 1ml of solution. 1μl of 1M levamisole was added upon repeating the experiment in an attempt to block endogenous alkaline phosphatase. Secondly, purchasable NBT/BCIP tablets were used, and dissolved in distilled water (the resulting concentrations were comparable) with 1μl of 1M levamisole.

3) Two different types of aqueous mountant were used: Immu-mount polyvinyl alcohol resin, Shandon, Pittsburg, USA, and Histotec permanent aqueous mountant (Serotec).

4) Once the cover slips were mounted, slides were initially dried on a 37°C hotplate (said to aid solidification) and subsequently on the bench at room temperature.

4.4 Assessment of immunohistochemistry for EGFR

A semi-quantitative grading scale was used to assess the quantity and distribution of EGFR expression in each slide. Slides were scanned by light microscopy (LJM and 50% independently by a Consultant Histopathologist) at low power (x40 and x100) to determine the overall percentage of positively staining tumour cells, stromal staining and tumour necrosis. High power field was used (x200) to examine the percentage membranous and cytoplasmic distribution and intensity of EGFR. Median percentage membranous and cytoplasmic staining values were calculated.
5. METHODS FOR MATRIX METALLOPROTEINASE-2

5.1 Immunohistochemical detection kit

The Dextran Polymer Conjugate Two-step Visualisation system Envision (DAKO, Ely, UK) was used.

5.2 Immunohistochemical method

Two litres of TBS buffer were prepared (see Standard Operating Procedure 8.4.1.)
Racked slides were placed in a 60° oven for 5 minutes to melt the embedding wax.

Twenty-five racked slides were dewaxed in xylene (Genta Medical) and rehydrated
by periodic agitation for 2 minutes through a series of alcohol solvents (99% and 95%
industrial methylated spirit, Genta Medical) under a fume hood, and washed in cold
running tap water.

Antigen retrieval using the pressure cooker (Standard Operating Procedure 8.4.1) was
performed for 2 minutes. The racked slides were placed in an appropriately sized
plastic container, cooled in water and bathed in TBS for 5 minutes on a magnetic
stirrer. Upon completion of the wash, excess buffer was wiped away from the corners
of each slide with soft tissue paper and then aligned in a humid chamber, to prevent
the sections drying out. Endogenous peroxidase was blocked for 30 minutes using
100 µl of 0.5% hydrogen peroxide from the Envision kit, followed by 5 minutes
buffer wash.
100 μl of anti MMP-2 primary antibody were applied using a calibrated Gilson’s pipette onto each slide and incubated for 60 minutes in the humid chamber at room temperature. Primary antibody was substituted by TBS for the negative control. The slides were washed in buffer and 100 μl of secondary polymer (Bottle 2) were applied to each slide for 30 minutes. Bathing in TBS followed for 5 minutes. The substrate-chromogen solution was prepared by mixing 1ml of buffered substrate solution (Bottle 3a) and 1 drop of 3,3’ diaminobenzidine chromogen solution, DAB (Bottle 3b) per 10 slides as supplied in the kit. 100 μl of substrate-chromogen solution were applied per slide, using the plastic Pasteur pipette supplied, and incubated for 10 minutes.

The slides were washed in cold running tap water for 5 minutes and counterstained with Mayer’s Haematoxylin for 15 seconds, placed in running water until the water ran clear and dehydrated using graded alcohol solvents. Cover slips were mounted in a fume cupboard by applying one drop of DPX (BDH Chemicals, England) obtained by immersing the tip of an orange stick into the flask containing the solution, onto a cover slip and laying the slide, tissue side down onto the cover slip. The loosely adhered cover slip was then fixed into position with gentle pressure using another orange stick. The slides were allowed to dry.

5.3 Method optimisation: Immunohistochemical staining methods

Different antigen retrieval techniques and antibody dilutions were utilised.

Antigen retrieval techniques:

1) No retrieval

2) Microwave in citrate buffer for 15 minutes
3) Pressure cook in microwave for 4 minutes

4) Pressure cook in citrate buffer for 2 minutes

Antibody dilutions:

- 1:50, 1:100, 1:150

5.4 Assessment of immunohistochemistry for MMP-2

A semi-quantitative grading scale was used to assess the quantity and distribution of MMP-2 expression in each slide. Slides were scanned by light microscopy at low power (x40 and x100) and high power (x200) to determine the overall percentage of positively staining tumour cells and the extent of stromal staining. A scale of zero to +++ was used to determine the extent of staining in the stroma, where 0 represented no staining, and +++ very extensive staining. Median percentage tumour cell staining and stromal staining values were calculated.
6. METHODS FOR MATRIX METALLOPROTEINASE-9

6.1 Immunohistochemical detection kit

The Dextran Polymer Conjugate Two-step Visualisation system Envision (DAKO, Ely, UK) was used.

6.2 Immunohistochemical method

Two litres of TBS buffer were prepared (see Standard Operating Procedure 8.1).

Racked slides were placed in a 60° oven for 5 minutes to melt the embedding wax.

Twenty-five racked slides were dewaxed in xylene (Genta Medical) and rehydrated by periodic agitation for 2 minutes through a series of alcohol solvents (99% and 95% industrial methylated spirit, Genta Medical) under a fume hood, and washed in cold running tap water.

Antigen retrieval using the pressure cooker (see Standard Operating Procedure 8.4.1) was performed for 2 minutes. The racked slides were placed in an appropriately sized plastic container, cooled in water and bathed in TBS for 5 minutes on a magnetic stirrer. Upon completion of the wash, excess buffer was wiped away from the corners of each slide with soft tissue paper and then aligned in a humid chamber, to prevent the sections drying out. Endogenous peroxidase was blocked for 30 minutes using 100 μl of 0.5% hydrogen peroxide (sufficient to cover the entire specimen on the slide) from the Envision kit, followed by 5 minutes buffer wash.
100 µl of primary antibody were applied using a calibrated Gilson’s pipette onto each slide and incubated for 30 minutes in the humid chamber at room temperature.

Primary antibody was substituted by TBS for the negative control. The slides were washed in buffer and two drops of secondary polymer (Bottle 2) were applied to each slide for 30 minutes. Bathing in TBS followed for 5 minutes.

The substrate-chromogen solution was prepared by mixing 1ml of buffered substrate solution (Bottle 3a) and 1 drop of 3,3’ diaminobenzidine chromogen solution, DAB (Bottle 3b) per 10 slides as supplied in the kit. 100 µl of substrate-chromogen solution were applied per slide, using the plastic Pasteur pipette supplied, and incubated for 10 minutes. The slides were washed in cold running tap water for 5 minutes and counterstained with Mayer’s Haematoxylin for 15 seconds, placed in running water until the water ran clear and dehydrated using graded alcohol solvents. Cover slips were mounted in a fume cupboard by applying one drop of DPX (BDH Chemicals, England).

6.3 Method optimisation

Different antigen retrieval techniques and antibody dilutions were utilised.

6.4 Assessment of immunohistochemistry for MMP-9

A semi-quantitative grading scale was used to assess the quantity and distribution of MMP-9 expression in each slide. Slides were scanned by light microscopy at low
power (x40 and x100) and high power (x200) to determine the overall percentage of positively staining tumour cells and the extent of stromal staining. Stromal staining was scored as 0 = negative, + = low, ++ = moderate and +++ = extensive. Median percentage tumour cell staining and stromal staining values were calculated.

7. METHODS FOR HYPOXIA INDUCIBLE FACTOR-1

7.1 Immunohistochemical detection kits

The Catalysed Signal Amplification (CSA) system and the CSA Ancillary System and the Envision visualisation systems (DAKO, Ely, UK) were used.

7.2 Immunohistochmical methods

7.2.1 Catalysed Signal Amplification (CSA) system

800mls TBST buffer were prepared (Standard Operating Procedure 8.3). The antigen retrieval solution was decanted into a Coplin jar, which was then placed into a thermostatic bath (Grant Instruments, UK) and heated to a temperature of 97°C (Standard Operating Procedure 8.4.2). Ten racked slides were dewaxed in xylene and rehydrated by periodic agitation through graded alcohol solvents (99% and 95%) and washed in cold running tap water.

The slides were submersed back-to-back in the solution using forceps for 45 minutes.
The temperature of the water bath was initially verified every ten minutes to ensure a constant temperature of 97°C. The slides were then carefully removed from the coplin jar, racked and bathed in TBST buffer for 5 minutes.

Slides were incubated for 10 minutes at room temperature in 100μl of avidin from the biotin blocking system (Bottle 1, CSA Ancillary System), and washed in TBST for three minutes. The slides were incubated for a further 10 minutes in 100μl of biotin (Bottle 2, CSA Ancillary System), followed by a three-minute wash in TBST.

100μl of endogenous peroxidase block was applied per slide in a humid chamber (Bottle 1) for 5 minutes, followed by a buffer wash.

100μl of protein block (Bottle 2) were applied to each slide for 5 minutes and 100 μl HIF-1α67 (primary antibody) immediately applied, without buffer wash, for overnight incubation at 4°C. Primary antibody was substituted by antibody diluent with background reducing components (CSA Ancillary System) for the negative control.

The following day, the streptavidin-biotin-peroxidase complex was prepared following the instructions provided in the CSA kit (bottles 5, 6 and 7) and allowed to stand for 30 minutes.
Table 2.6 Streptavidin-biotin-peroxidase complex preparation

<table>
<thead>
<tr>
<th>Bottle number</th>
<th>1-10</th>
<th>11-20</th>
<th>21-30</th>
<th>31-40</th>
<th>41-50</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>1ml</td>
<td>2ml</td>
<td>3mls</td>
<td>4mls</td>
<td>5mls</td>
</tr>
<tr>
<td>5</td>
<td>1 drop</td>
<td>2 drops</td>
<td>3 drops</td>
<td>4 drops</td>
<td>5 drops</td>
</tr>
<tr>
<td>6</td>
<td>1 drop</td>
<td>2 drops</td>
<td>3 drops</td>
<td>4 drops</td>
<td>5 drops</td>
</tr>
</tbody>
</table>

The primary antibody was washed from the slides for 5 minutes and 100 μl of biotinylated link antibody per slide (bottle 4) incubated for 15 minutes at room temperature.

Following buffer wash, 100 μl of streptavidin-biotin-peroxidase complex were pipetted onto each slide for 15 minutes and washed. 100 μl of streptavidin-peroxidase (Bottle 9) were applied for 15 minutes.

The substrate-chromogen solution was prepared by using 10 drops of substrate tris buffer concentrate (bottle 11) in 10mls distilled water, one substrate tablet (Bottle 10) and 1 drop of substrate hydrogen peroxide (bottle 12) for every 2ml of substrate mixture (Table 2.8).

Two-three drops of the substrate-chromogen solution were incubated per slide for 5 minutes and washed in cold running water.
### Table 2.7 Substrate-chromogen solution preparation

<table>
<thead>
<tr>
<th></th>
<th>1-20 slides</th>
<th>21-40 slides</th>
<th>41-60 slides</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bottle 11</td>
<td>10 drops</td>
<td>10 drops</td>
<td>10 drops</td>
</tr>
<tr>
<td>Distilled water</td>
<td>10mls</td>
<td>10mls</td>
<td>10mls</td>
</tr>
<tr>
<td>Bottle 10</td>
<td>1 tablet</td>
<td>1 tablet</td>
<td>1 tablet</td>
</tr>
<tr>
<td>Millilitres of solution</td>
<td>2</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>Bottle 12</td>
<td>1 drop</td>
<td>2 drops</td>
<td>3 drops</td>
</tr>
</tbody>
</table>

The slides were submersed in Mayer’s Haematoxylin for 15 seconds and thoroughly washed in cold running water until the latter was clear.

The slides were dehydrated through graded alcohols and mounted using DPX (BDH Chemicals, England).

#### 7.2.2 Envision system

2L of TBS buffer was prepared (Standard Operating procedure 8.1).

The positive control was dewaxed in xylene and rehydrated by periodic agitation through graded alcohol solvents (99% and 95%), washed in cold running tap water and bathed in buffer.

Antigen retrieval was undertaken in the same manner described for the CSA method (*vide supra*) and the slides bathed in buffer.
Upon completion of the wash, excess buffer was wiped away from the corners of each slide, which were then placed in a humid chamber for incubation.

100 µl of 0.5% hydrogen peroxide (Bottle 1) were applied per slide for 5 minutes in the humid chamber followed by 5 minutes buffer wash.

100 µl of normal rabbit serum, diluted to a 1:20 concentration in TBS were pipetted onto each slide for 15 minutes, to block background staining. Buffer wash followed.

100 µl of HIF-1α67 (primary antibody) antibody (Novocastra, UK) were applied using a calibrated Gilson’s pipette onto each slide and incubated for 60 minutes at room temperature. Primary antibody was substituted by TBS for the negative control.

The slides were washed in buffer and two drops of secondary polymer (Bottle 2, Envision, DAKO, UK) were applied to each slide in the humid chamber for 30 minutes. Bathing in TBS followed for 5 minutes.

The substrate-chromogen solution was prepared by mixing 1ml of buffered substrate solution (Bottle 3a) and 1 drop of DAB+chromogen (Bottle 3b) per 10 slides as supplied in the kit. Three drops of substrate-chromogen solution were applied per slide and incubated in the humid chamber for 5 minutes.

The slides were washed in cold running water for 2 minutes, counterstained with Mayer’s haematoxylin for 15 seconds, and washed again thoroughly in cold running water until it ran clear.
The slides were dehydrated using graded alcohol solvents, and cover slips were mounted using DPX (BDH Chemicals, England).

8. STANDARD OPERATING PROCEDURES FOR REAGENTS USED IN IMMUNOHISTOCHEMISTRY

8.1 Tris-buffered saline for use with the Envision visualisation system

Constitution:  
0.05M Tris  
0.15M NaCl

Reagents per litre:  
6g powdered Tris (Roche Diagnostics, Indianapolis, USA)  
4.38g NaCl (Roche Diagnostics, Indianapolis, USA)  
1 litre distilled water

pH adjustment:  
calibrated pH meter (Hanna Instruments, UK)  
3M HCl (Fisher Chemicals, UK)  
3M NaOH (Fisher Chemicals, UK)

Quantity required:  
2 litres for 25 slides

8.1.1 Method

50mls distilled water were placed in a 1L beaker with a flea on a magnetic stirrer.

Powdered Tris was weighed on a balance to the correct quantity and voided into the beaker. The solution was stirred until the Tris was fully dissolved.

The correct quantity of sodium chloride was then added to the beaker and stirred until fully dissolved.
The pH of the solution was measured using the calibrated pH meter and adjusted to 7.6 by carefully decanting hydrochloric acid (3M) from a small glass beaker. Sodium hydroxide (3M) was utilised to revert the hydrochloric acid, if the pH recorded below 7.6.

The solution was made-up to 1L with distilled water and decanted into a 1L flask with a lid for storage to a maximum of six months.

8.2 Citric acid buffer

Constitution: 10mM citric acid (x20 concentrated)

Reagents:
- 42g citric acid monohydrate (AnalR)
- 500 mls distilled water
- Sodium hydroxide pellets (Fisher Chemicals, UK)

pH adjustment:
- calibrated pH meter (Hanna Instruments)
- 3M HCl (Fisher Chemicals, UK)
- 3M NaOH (Fisher Chemicals, UK)

Quantity required: 75mls in 1425mls distilled water for a 1.5L capacity pressure cooker.

8.2.1 Method

The citric acid was dissolved in the distilled water in a beaker on a magnetic stirrer. The pH was adjusted using the calibrated pH meter to 6.0 using NaOH pellets. The volume was made-up to 1L using distilled water in a 1L flask.
To obtain a 0.01 M solution for use, a dilution of 1:20 was utilised; thus for a 1.5 L capacity pressure cooker, 75 mls citric acid buffer and 1425 mls distilled water was required.

### 8.3 Tris buffered saline with Tween 20 for use with the CSA system

Concentrate (x10) available from DAKO, either separately or in the CSA Ancillary System.

**Constitution:**

- 0.05 M Tris
- 0.30 M NaCl
- 0.1% Tween 20

**pH adjustment:**

- calibrated pH meter (Hanna Instruments)
- 3 M HCl (Fisher Chemicals, UK)
- 3 M NaOH (Fisher Chemicals, UK)

**Quantity required:**

- 1:10 dilution of concentrated solution to distilled water
- 40 mls of solution for use in one coplin jar
- 4 mls solution to 36 mls distilled water
- Total volume 800 mls to allow for 20 washes

### 8.4 Antigen Retrieval Techniques

#### 8.4.1 Pressure cooking

10 mM citric acid was used as the target retrieval buffer (SOP 8.2).

The slides were positioned in alternate slots in a slide rack and rinsed in distilled water.
1.5L of 10mM citric acid buffer was decanted into a Prestige (model 6189) pressure cooker and allowed to boil with the lid unlocked on a hot plate.

Once the buffer was boiling, with the steam regulator at the one dot position, the slides were lowered into the buffer with long forceps and the lid sealed. The small pressure indicator then rose. Once the large pressure indicator rose, the hot plate was switched off (as sufficient latent heat enabled pressurisation to be maintained) and the slides were pressure cooked for 2 minutes. The pressure cooker was then placed in the cold water-filled sink until the small pressure indicator returned to its original position, enabling the lid to be opened. The slides were retrieved with long forceps and washed in cold running tap water, followed by equilibration in TBS for 5 minutes.

8.4.2 Thermostatic bath

Slides were heated using a thermostatic water bath in target antigen retrieval solution. A standard Coplin jar (maximum 10 slides) allows for 40mls antigen retrieval:

4mls of x10 concentrated target retrieval solution (DAKO, Ely, UK) were pipetted into the coplin jar.

36mls distilled water were added and stirred using a flea on a magnetic stirrer. The pH was verified using the calibrated pH meter and adjusted to 6.1 using concentrated hydrochloric acid (Fisher Chemicals, UK) as described before.
9. SUMMARY OF METHODS USED IN PREVIOUS WORK TO EVALUATE MVD AND VEGF

9.1 Direct measurement of angiogenesis: Microvessel density (MVD)

In order to facilitate the laborious task of counting microvessels identified by pan-endothelial markers, a semi-automated, computer assisted image analysis microvessel counting method to assess tumour microvasculature, was devised by Goddard and Sutton et al (Goddard et al., 2002). Images from paraffin embedded tissue block sections from liver metastases and superficial bladder cancer, stained with CD34, were captured using a colour video camera and a Scion cg-f frame grabber attached to an Apple MacIntosh G-3 computer. A macro was written which automatically converted the captured coloured images to grey scale and counted the number of individually stained vessels. Both the hotspots and the random sampling counting techniques were employed. Using this method, tumour edge microvessel hotspots, in the same series of colorectal liver metastases as this thesis has employed, were found to be significantly associated with poor prognosis (“Angiogenesis in Resected Colorectal Liver Metastases”, CD Sutton 2004).

9.2 Indirect measurement of angiogenesis: Angiogenesis-modulating cytokines

Since angiogenesis is promoted by pro-angiogenic factors (Folkman and Klagsbrun, 1987) and inhibited by anti-angiogenic factors, measuring the protein or gene expression of these factors can be an indirect measure of angiogenesis.
Pro-angiogenic growth factors either directly stimulate endothelial cells to proliferate or encourage the release of further cytokines from host cells.

Quantification of biochemical markers of angiogenesis can help elucidate the biological behaviour of individual tumours, help understand the mechanisms of tumour formation, growth and metastasis and help in identifying targets for novel chemopreventative/therapeutic agents.

The table below summarises the materials used for the immunohistochemical detection of VEGF:

**Table 2.8 Immunohistochemistry for VEGF**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>VG1 (Gift from Oxford)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Dilution</strong></td>
<td>1:2</td>
</tr>
<tr>
<td><strong>Detection kit</strong></td>
<td>Envision, Dako, Ely, UK</td>
</tr>
<tr>
<td><strong>Incubation</strong></td>
<td>30 minutes at room temperature</td>
</tr>
</tbody>
</table>
CLINICO-PATHOLOGICAL DATA
1. PATIENT DEMOGRAPHICS

A total of 197 patients underwent a liver resection for colorectal liver metastases at the University Hospitals of Leicester and the Royal Liverpool Hospital during the study period. The following exclusion criteria were applied: 30-day mortality (n=7) and insufficient or inadequate pathological archival tissue blocks (n=9). 181 patients (male n=121, female n=60) were therefore suitable for inclusion in the study, aged between 25 years and 81 years (mean 61 years).

2. CONSENT

Twenty-three patients were identified from the University Hospitals of Leicester database to have been alive with recent documented attendance to an outpatient clinic. These patients were directly contacted for consent. One patient was listed as too unwell to attend outpatients, and was thus not contacted.

Thirty-one letters were sent to General Practitioners to ascertain whether patients were alive and if so, suitable to be approached for consent. Eight patients were deemed eligible for consent, as were thus sent letters. Four patients had moved practice and were thus un-contactable.

A total of 31 patients were approached for consent. 30 replies were received. There was one non-responder despite a follow-up letter. One patient returned the form without signature but with no clear objection, and was subsequently unable to be contacted. No patients denied the use of tissue for research purposes.
3. SURVIVAL

Patient survival (Table 6), measured from the date of hepatic resection to either the date of death or the current date, ranged from 48 to 3573 days (median 1410 days). All recorded deaths were cancer-specific.

Table 3.1 Patient survival data

<table>
<thead>
<tr>
<th>Number of patients (%)</th>
<th>Alive at 2 years</th>
<th>Alive at 5 years</th>
<th>Overall survival</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>136 (75)</td>
<td>59 (32.6)</td>
<td>107 (59%)</td>
</tr>
</tbody>
</table>

4. CLINICO-PATHOLOGICAL VARIABLES

The following clinicopathological variables were included (Table 3.2).

Table 3.2 Clinicopathological factors and their prognostic value

<table>
<thead>
<tr>
<th>Clinicopathological factor</th>
<th>Number of patients</th>
<th>Survival (log rank test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dukes’ stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>n=6</td>
<td>P=0.45</td>
</tr>
<tr>
<td>B</td>
<td>n=79</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>n=96</td>
<td></td>
</tr>
<tr>
<td>Disease distribution</td>
<td></td>
<td></td>
</tr>
<tr>
<td>unilobar</td>
<td>n=109</td>
<td>P=0.55</td>
</tr>
<tr>
<td>bilobar</td>
<td>n=72</td>
<td></td>
</tr>
<tr>
<td>Metastases</td>
<td></td>
<td></td>
</tr>
<tr>
<td>synchronous</td>
<td>n=88</td>
<td>P=0.73</td>
</tr>
<tr>
<td>metachronous</td>
<td>n=93</td>
<td></td>
</tr>
<tr>
<td>Metastases</td>
<td></td>
<td></td>
</tr>
<tr>
<td>range</td>
<td>1-13</td>
<td>P=0.75</td>
</tr>
<tr>
<td>median</td>
<td>n=2</td>
<td></td>
</tr>
<tr>
<td>1-3 lesions</td>
<td>n=145</td>
<td></td>
</tr>
<tr>
<td>&gt;3 lesions</td>
<td>n=36</td>
<td></td>
</tr>
<tr>
<td>Metastases</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 lesion</td>
<td>n=75</td>
<td>P=0.49</td>
</tr>
<tr>
<td>&gt;1 lesion</td>
<td>n=106</td>
<td></td>
</tr>
<tr>
<td>Size of lesion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>range</td>
<td>4 - 230mm</td>
<td>P=0.42</td>
</tr>
<tr>
<td>median</td>
<td>40mm</td>
<td></td>
</tr>
<tr>
<td>&lt;50mm diameter</td>
<td>n=127</td>
<td></td>
</tr>
<tr>
<td>&gt;50mm diameter</td>
<td>n=54</td>
<td></td>
</tr>
</tbody>
</table>
5. DISCUSSION

This discussion aims to briefly summarise the clinico-pathological variables analysed. Please see “Angiogenesis in Resected Colorectal Liver Metasases” (Sutton, 2004) for a full review of these factors in this series.

Patients undergoing a liver resection for colorectal liver metastases have an estimated 5-year survival of 25-35%. The survival figures obtained from this study demonstrated a 32.6% 5-year survival rate, with 75% of patients surviving 2 years, and a 30-day mortality of 3.9%, which is in keeping with the literature. The mean survival was 4 years.

Predicting the outcome following resection of colorectal liver metastases has been investigated by a number of studies, most notably by Nordlinger (Nordlinger et al., 1996) and Fong (Fong et al., 1999), who reported the two largest series of factors related to outcome following hepatectomy. These authors examined numerous patient and tumour characteristics and identified those associated with poor patient prognosis.

The principal operative factor of prognostic significance in Fong’s series was the extent of liver resection. Patients who had a single lobe excised had improved survival when compared with patients who had more than one lobe excised, adjusted for post-operative mortality (5-year survival 40% and 33% respectively) (Fong et al., 1999). In Nordlinger’s study, however, the extent of resection only had an impact on post-operative mortality and not on 5-year survival figures (Nordlinger et al., 1996).
More recently, Adam et al correlated the number of resected segments (greater or equal to 3) to poorer outcome (p=0.02) in a consecutive series of 1439 patients (Adam et al., 2004a).

The main primary tumour characteristic associated with poor prognosis was the presence of lymph node metastases (Nordlinger et al., 1996) (Fong et al., 1999). Synchronous liver metastases detected less than 12 months after colorectal resection were associated with poorer patient survival as compared with metastases detected 12 months after colorectal resection (p=0.01) (Fong et al., 1999). Patients presenting within 24 months of colorectal resection had a worse outcome in Nordlinger’s series (Nordlinger et al., 1996).

Pre-operative carcinogen embryonic antigen (CEA) levels and CA 19-9 have also been demonstrated as markers of poor survival. CEA >200ng/ml (Fong et al., 1999) and >30mg/ml (Nordlinger et al., 1996) were statistically correlated to poor survival. CA 19-9 >100UI/L in Adam’s study was negatively prognostic (Adam et al., 2004a).

Patients with a solitary metastasis have a survival advantage over patients with multiple metastases (Adam et al., 2004a) (Adam et al., 2004b) (Nordlinger et al., 1996) (Fong et al., 1999). Adam et al, from Bismuth’s group in Paris, reported a significant association between the number of liver metastases at operation (p=0.003) and the number of metastases in the resection specimen (p=0.02).

The size of the metastatic deposit has also been shown to be negatively prognostic. Metastatic deposits measuring greater than 5cm diameter are also associated with a
worse outcome in the original, larger series (Nordlinger et al., 1996) (Fong et al., 1999). This finding has been more recently confirmed by Bismuth’s team, who reported poorer survival figures in association with maximum tumour diameter (range 10-160mm, median diameter 38 mm) (Adam et al., 2004b).

The strongest predictor of recurrence was hepatic lymph node involvement and disease at the resection margin in both Fong’s and Nordlinger’s work, and disease-free survival in Adam’s study (Adam et al., 2004a) (Nordlinger et al., 1996) (Fong et al., 1999). Only 20% of patients with a positive resection margin survived 5 years in Fong’s series (Fong et al., 1999).

Since a significant correlation has been demonstrated between certain clinico-pathological factors, principally, size and number of metastases, disease distribution, stage of primary tumour, disease free interval and resection margin and poor survival, pre-operative models to predict survival and clinical risk scores have been developed.

Fong and Blumgart’s group devised a clinical risk score (CRS) to predict outcome in patients with colorectal liver metastases. Each of the following factors was allocated one point to generate the score: lymph node positive primary tumour, disease free interval of less than 12 months, more than one hepatic tumour, maximum tumour diameter of >5cm and CEA of >200ng/mL. Scores between 0-2 were associated with 5-year survival rates of 52.3%, and scores between 3 and 5 with 20.2%. This scoring system was more recently used to identify patients who would benefit from a pre-hepatectomy laparoscopy in order to identify radiographically undetected unresectable disease. Patients with a CRS of 0-1 did not have undetected resectable disease at
laparoscopy, and thus underwent resection; patients with a CRS of 2-3 had unresectable disease in 11% of cases, patients with a CRS of 4 or 5 had unresectable disease in 24% of cases, and were thus precluded from a major laparotomy (Grobmyer et al., 2004). In this context, the authors supported the use of CRS to identify patients who might benefit from a diagnostic laparoscopy (Grobmyer et al., 2004). Mann et al deemed the CRS as highly predictive of survival, albeit in a much smaller series of 77 patients (Mann et al., 2004).

Adam et al assigned four factors in their pre-operative model to predict survival, namely a rectal primary, greater than three metastases, maximum tumour diameter > 10cm and CA 19-9>100UI/L (Adam et al., 2004a). Patients with no risk factors had a 5-year survival of 59%, mean survival of 30% with one factor, mean survival of 7% with two factors and survival of 0-1% with 3 and 4 factors. Disease-free survival was 42% without any risk factors, 10-16% with for one factor, 0-1% for two factors and 0% for 3 and 4 factors (Adam et al., 2004a).

This study, however, mostly failed to reproduce such associations, probably due to the smaller study population (Fong and Nordlinger evaluated 1001 and 1568 patients respectively (Fong et al., 1999) (Nordlinger et al., 1996), whereas this study examined 181 patients) although it was interesting that bilobar disease, although not predictive of overall survival, was statistically correlated with poor 5-year survival (p=0.01). Perhaps a larger study population could have yielded statistically significant results.

Disease recurrence affected 61% of patients in this study, comparable to 55% documented by Nordlinger (Nordlinger et al., 1996), 45% of these occurring in the
liver (n=50). It is difficult however to postulate whether the documented recurrence was true recurrence or simply a reflection of disease progression secondary to micrometastases not detected at initial imaging or at operation, despite intra-operative ultrasound scanning.
CARBONIC ANHYDRASE IX
1. IMMUNOHISTOCHEMICAL EXPRESSION OF CAIX

The recommendation of 1:20 was the first dilution used, followed by 1:25, 1:100 and 1:200. Upon review by a Consultant Histopathologist (Senior Lecturer with experience of CAIX grading) the recommended 1:20 dilution was used for all slides. CAIX immunoreactivity was detected in the cell membrane (mCAIX), in the cytoplasm (cCAIX) of tumour cells, and in the surrounding stroma (Figure 4.1). NSCLC was used as the positive control (Figure 4.2). Although a pattern of perinecrotic distribution was observed, it was not exclusively as such. CAIX was not consistently detected at the tumour edge.

Seventy four percent of patients (n=136) expressed mCAIX and 85% of patients (n=156) expressed cCAIX with a range of percentage tumour cell coverage of 0-100%, and median percentage expression of 10% and 20% respectively. Both mCAIX and cCAIX expression was found in 135 cases, twenty-one cases expressed only cCAIX, and only one case expressed only mCAIX. Twenty-four cases did not express CAIX (Table 4.1). Stromal CAIX was observed in 51 patients.

<table>
<thead>
<tr>
<th></th>
<th>Membranous CAIX (mCAIX)</th>
<th>Cytoplasmic CAIX (cCAIX)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of patients</td>
<td>181</td>
<td>181</td>
</tr>
<tr>
<td>Positive cases (%)</td>
<td>136 (74)</td>
<td>156 (85)</td>
</tr>
<tr>
<td>Negative cases (%)</td>
<td>45 (25)</td>
<td>25 (14)</td>
</tr>
<tr>
<td>Range of percentage expression (%)</td>
<td>0 – 100</td>
<td>0 – 100</td>
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<tr>
<td>Median percentage expression (%)</td>
<td>10</td>
<td>20</td>
</tr>
</tbody>
</table>
Figure 4.1 Membranous and cytoplasmic CAIX expression in a liver metastasis

(x200 magnification)

Figure 4.2 CAIX immunoreactivity in NSCLC

(x200 magnification)
Since tumour edge samples were used, tumour tissue abutted normal liver tissue comprising hepatocytes and epithelial bile ducts. Normal hepatocytes did not express CAIX (Figure 4.4) whereas strong immunoreactivity was detected in the epithelial cell-lined bile duct cells localised in normal liver tissue. Bile duct immunoreactivity therefore served as a reproducible, reliable internal control (Figure 4.5).
Figure 4.4 Tumour edge and normal liver tissue

Figure 4.5 CAIX immunoreactivity in bile ducts
Strong CAIX immunoreactivity was also detected in gallbladder specimens, as shown in Figure 4.6.

Figure 4.6 CAIX immunoreactivity in a gallbladder specimen

The majority of tumours in this series were characterised by extensive necrosis (n=103). Nine tumours did not have necrosis, 16 had minimal necrosis and 53 had moderate necrosis (Figure 4.7a-d).
Figure 4.7 Necrosis in liver metastases at x40 magnification

4.7a. Absent necrosis

4.7b. Minimal necrosis

4.7c. Moderate necrosis

4.7d. Extensive necrosis
1.1 Inter-observer variability

A semi-quantitative grading scale to interpret immunoreactivity was devised by a Consultant Histopathologist. Once the investigator had graded all the slides, the Consultant independently scored 50 different, randomly selected slides. In over 52% of slides, the percentage tumour coverage documented by the investigator was exactly the same as that documented by the Consultant. In 38% of cases, the difference in tumour coverage was within an acceptable 10% of each other, and within 10% of cases the difference was up to a maximum of 20% difference (Table 4.2). The slides with 20% difference were reviewed and agreed upon.

Table 4.2 Inter-observer variability

<table>
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<th>Inter-observer correlation</th>
<th>Consultant</th>
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<tr>
<td>Exact</td>
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</tr>
<tr>
<td>+/- 10% difference</td>
<td>19</td>
</tr>
<tr>
<td>+/-10-20% difference</td>
<td>5</td>
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</table>

2. CAIX AND CLINICOPATHOLOGICAL DATA

Significantly more mCAIX is present in synchronous lesions as compared with metachronous lesions (p=0.03). Otherwise, there was no statistical correlation between CAIX and the clinicopathological variables (Table 4.3).
3. CAIX AND ANGIOGENESIS

Two interesting findings were observed. Firstly, most protein expression of CAIX was concentrated away from the invasive tumour edge, which was the only area of microvessel density correlating with poor prognosis in this series (Sutton, 2004).

Secondly, CAIX did not statistically correlate with microvessel density (Table 4.4).

Table 4.3 CAIX expression and clinicopathological variables

<table>
<thead>
<tr>
<th>Pathological factor</th>
<th>Membranous CAIX</th>
<th>Cytoplasmic CAIX</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>&lt;median</td>
<td>≥median</td>
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<tr>
<td>Synchronicity</td>
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<tr>
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<td>Metachronous</td>
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<td>Disease distribution</td>
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<tr>
<td>Unilobar</td>
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<td>63</td>
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<td>Bilobar</td>
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<td>43</td>
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<tr>
<td>Size of lesion</td>
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<td></td>
</tr>
<tr>
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<td>&gt;3 lesions</td>
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Table 4.4 CAIX and MVD

<table>
<thead>
<tr>
<th>Microvessel Density</th>
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<th>cCAIX</th>
<th>p value</th>
<th>p value</th>
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<tr>
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<tr>
<td>Contiguous edge count</td>
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<tr>
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<tr>
<td>Below median</td>
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<td>Intra-tumour cumulative mean</td>
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4. CAIX AND PATIENT SURVIVAL

Figure 4.8 Median membranous CAIX expression and survival

Figure 4.9 Median liver cytoplasmic CAIX expression and patient survival
Patients with greater, or equal to the median expression of membranous CAIX had a significantly poorer outcome (p=0.02) as compared with those with less than the median CAIX expression (Figure 4.8). Although there was a similar trend with regards to cytoplasmic CAIX (Figure 4.9), it did not reach statistical significance (p=0.21).

Tumour necrosis was significantly associated with poor patient survival (p=0.0002) (Figure 4.10). None of the clinico-pathological factors described in Table 4.3 were associated with poor overall survival on univariate analysis.

5. DISCUSSION

CA IX is a transmembrane, acidic glycoprotein that catalyses the hydration of carbon dioxide into bicarbonate and a hydrogen ion (Opavský et al., 1996; Pastorek et al., 1994). It participates in the regulation of intracellular and extracellular acid-base balance, protecting tumour cells against apoptosis (Nogradi, 1998; Opavský et al., 1996; Saarnio et al., 1998; Karhumaa et al., 2001), and promoting lysis of the
extracellular matrix which enables cells to migrate and disseminate (Beasley et al., 2001).

CAIX expression has been detected in a perinecrotic tumour distribution (Ivanov et al., 2001) (Chia et al., 2001; Beasley et al., 2001; Hui et al., 2002; Loncaster et al., 2001) and correlated with areas of tumour hypoxia measured in vivo (Loncaster et al., 2001), hence CAIX has been proposed as an endogenous marker of hypoxia.

CAIX has been associated with poor prognosis in some primary tumours such as breast (Chia et al., 2001) non-small cell lung (Giatromanolaki et al., 2001; Swinson et al., 2003) and cervical cancer (Loncaster et al., 2001). However, a good prognosis has been observed in renal cell carcinoma (RCC) (Bui et al., 2003). In Bui’s study, patients with metastatic disease at diagnosis with low expression of CAIX had a median survival of 5.5 months, in contrast to patients with high expression of CAIX whose median survival was 24.8 months. No associations were found between CAIX expression and tumour stage and grade for patients with metastatic RCC. Low CAIX expression was independently associated with poor survival. In patients with non-metastatic disease, CAIX was not significantly associated with outcome. The discrepancy between studies regarding the prognostic significance of CAIX expression remains unclear; however, Bui et al suggest that it may relate to the precise role of CAIX in different tumours, by either influencing tumour behaviour or reflecting tumour progression.

In primary tumours, CAIX has been immunohistochemically detected in a membranous, perinecrotic distribution, often with highest expression closest to areas
of necrosis in head and neck, ovary, breast (Ivanov et al., 2001; Chia et al., 2001), head and neck squamous cell (HNSCC) (Beasley et al., 2001), nasopharyngeal (Hui et al., 2002), locally advanced squamous cell (Loncaster et al., 2001) and non-small cell lung cancer (Swinson et al., 2003), leading to the hypothesis that tumour hypoxia stimulates up-regulation of CAIX.

Only three studies were identified from the literature that investigated CAIX in metastatic lesions. One paper examined CAIX in two liver, one chest, one colon, one adrenal and nine lymph node metastases from renal clear cell carcinoma (Bui et al., 2003), the second also examined a liver metastasis from a colorectal primary (Saarnio et al., 1998), and the third examined CAIX in seven lymph nodes from head and neck squamous cell carcinoma (Beasley et al., 2001). Therefore, only 3 liver metastatic lesions, with only one from colorectal origin have been examined for CAIX. The results were a descriptive statement as to whether the patterns of expressions resembled those encountered in the respective primary tumours. All the remaining published data on CAIX has focused on its biological activity in primary tumours and potential influence on patient prognosis.

This is the first series to date to study the clinical relevance of CAIX in metastatic disease. CAIX immunoreactivity in liver metastases was clearly observed in this study. Both membranous and cytoplasmic expression of CAIX was present in 135 patients (75%). Twenty-four metastases did not express any CAIX (13%). Of the remaining 22 metastases, one expressed membranous CAIX and the other cytoplasmic CAIX (n=21). In a proportion of cases, (n=51, 28%) CAIX was detected
in the stroma, the significance of which is unclear, but believed to be as a result of CAIX expression in fibroblasts.

Although CAIX expression appears to be associated principally with poor survival in primary tumours, not all studies have documented similar findings. Some authors have documented trends and/or significant associations between increasing expression of CAIX and poor prognosis, such as in NSCLC (Swinson et al., 2003) (Giatromanolaki et al., 2001), locally advanced carcinomas of the cervix (Loncaster et al., 2001) and invasive breast carcinoma (Chia et al., 2001). Another study reported no individual association between CAIX and prognosis but, describe worse prognosis when CAIX is considered together with other variables associated with hypoxia and angiogenesis (Hui et al., 2002), and in one study of advanced clear cell carcinoma, low CAIX expression correlated with poor survival in patients with metastatic disease at presentation (Bui et al., 2003) and failed to correlate with survival in patients with non-metastatic disease. In this study, equal or greater than the median expression of membranous CAIX expression, was significantly associated with poor overall survival on univariate analysis (p=0.02). A similar trend was observed for cytoplasmic CAIX, but failed to reach statistical significance.
EPIDERMAL GROWTH FACTOR
RECEPTOR
1. OPTIMISATION OF IMMUNOHISTOCHEMISTRY

1.1 ChemMate system (DAKO, Ely, UK)

Despite using different antibody concentrations (1:10; 1:20; 1:50), extensive background and endogenous staining throughout the liver tissue section were obtained. Thus, the percentage tumour staining could not be specifically or reliably interpreted. Upon review with the Consultant Histopathologist, this method was disregarded.

1.2 The avidin biotin alkaline phosphatase (ABC) method (DAKO, Ely, UK)

This method produced an unreliable staining pattern after 40 minutes incubation, as background staining from endogenous biotin and alkaline phosphatase was detected in the liver tissue sections.

1.3 EnVision detection system (DAKO, Ely, UK)

Clear staining was obtained, as this method did not rely on biotin or alkaline phosphatase-based antibodies. Primary antibody concentration of 1:20 was used. After the slides were reviewed by the Consultant Histopathologist, this method was selected.
2. IMMUNOHISTOCHEMICAL EXPRESSION OF EGFR

Upon microscopy at varying magnification (x40, x100, x200 and x400), EGFR was detected within tumour cells (cytoplasmic EGFR) and on the cell membrane (membranous EGFR), as illustrated below (Figure 5.1). Any staining was considered positive. EGFR expression was detected in 62/181 cases (34%). Of these positive cases, 11% (n=20) expressed only membranous EGFR, 12% (n=22) only cytoplasmic EGFR and 11% (n=20) co-expressed both membranous and cytoplasmic EGFR. Positive and negative controls in NSCLC are shown in Figures 5.2 and 5.3.

Figure 5.1 EGFR immunoreactivity in a colorectal liver metastasis

(x200 magnification)
Figure 5.2 EGFR immunoreactivity in NSCLC

(x200 magnification)

Figure 5.3 Negative EGFR immunoreactivity in NSCLC

(x100 magnification)
Membranous expression was detected in 40 cases and cytoplasmic in 42 cases. There was no specific distribution of staining within the section of tumour. Percentage expression of mEGFR and cEGFR was divided into present versus absent staining, and compared with the clinico-pathological variables, CAIX, MVD and survival.

3. EGFR AND THE CLINICOPATHOLOGICAL DATA

No significant correlations were found (Table 5.1).

Table 5.1 Clinico-pathological variables and EGFR

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<th>Pathological factor</th>
<th>Megfr absent</th>
<th>Megfr present</th>
<th>P value</th>
<th>cEGFR absent</th>
<th>cEGFR present</th>
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<tr>
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<tr>
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4. EGFR AND MICROVESSEL DENSITY

Membranous EGFR and cytoplasmic EGFR did not correlate with MVD (Table 5.2).

Table 5.2 EGFR and MVD

<table>
<thead>
<tr>
<th>Microvessel Density</th>
<th>MEGFR</th>
<th>P value</th>
<th>cEGFR</th>
<th>P value</th>
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<tr>
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<td>absent</td>
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<tr>
<td>Below median</td>
<td>64</td>
<td>20</td>
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<td>Above median</td>
<td>47</td>
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<tr>
<td>Contiguous edge count</td>
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<td></td>
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</tr>
<tr>
<td>Below median</td>
<td>60</td>
<td>16</td>
<td>0.2</td>
<td>59</td>
</tr>
<tr>
<td>Above median</td>
<td>51</td>
<td>22</td>
<td>0.74</td>
<td>55</td>
</tr>
<tr>
<td>Intra-tumour hotspot</td>
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</tr>
<tr>
<td>Below median</td>
<td>75</td>
<td>17</td>
<td>0.23</td>
<td>69</td>
</tr>
<tr>
<td>Above median</td>
<td>66</td>
<td>23</td>
<td>0.56</td>
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<tr>
<td>Intra-tumour cumulative mean</td>
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<tr>
<td>Below median</td>
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<td>17</td>
<td>0.74</td>
<td>59</td>
</tr>
</tbody>
</table>

5. RELATIONSHIP BETWEEN EGFR, CAIX AND VEGF

No relationship between EGFR, CAIX and VEGF was found (Table 5.3).

Table 5.3 EGFR, CAIX and VEGF

<table>
<thead>
<tr>
<th></th>
<th>mEGFR</th>
<th>P value</th>
<th>cEGFR</th>
<th>P value</th>
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<tr>
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<tr>
<td>Membranous CAIX</td>
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<td></td>
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</tr>
<tr>
<td>Below median</td>
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<td>Above median</td>
<td>81</td>
<td>25</td>
<td>0.39</td>
<td>79</td>
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<tr>
<td>Cytoplasmic CAIX</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Below median</td>
<td>65</td>
<td>16</td>
<td>0.49</td>
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<td>Above median</td>
<td>76</td>
<td>24</td>
<td>0.9</td>
<td>77</td>
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<tr>
<td>Stromal CAIX</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Below median</td>
<td>100</td>
<td>30</td>
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<tr>
<td>Above median</td>
<td>41</td>
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<td>0.39</td>
<td>37</td>
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<tr>
<td>Tumour cell VEGF</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Below median</td>
<td>73</td>
<td>17</td>
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<td>72</td>
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<tr>
<td>Above median</td>
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<td>0.3</td>
<td>67</td>
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<tr>
<td>Stromal VEGF</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Below median</td>
<td>66</td>
<td>23</td>
<td>0.2</td>
<td>70</td>
</tr>
<tr>
<td>Above median</td>
<td>75</td>
<td>17</td>
<td>0.56</td>
<td>69</td>
</tr>
</tbody>
</table>
6. EGFR AND PATIENT SURVIVAL

Patients with tumours expressing membranous EGFR had a significantly poorer outcome (p=0.01) as compared with those without EGFR expression (Figure 5.4). Although there was a similar trend with regards to cytoplasmic EGFR (Figure 5.5), it did not reach statistical significance (p=0.25).

11. DISCUSSION

EGFR is a transmembrane glycoprotein tyrosine kinase receptor of 170kDa implicated in cellular signalling events important for the control of cell growth and differentiation (Carter and Kung, 1994). EGFR promotes transcription of genes involved in the control of cellular proliferation through the MAP (Brown, 1995; Prenzel et al., 2001) and JAK/STAT pathway (Brown, 1995) and promotes cell survival by activating anti-apoptotic factors via the phosphatidylinositol-3-kinase pathway. EGFR is an important constitutive component of normal human tissue both during embryogenesis and throughout maturation (Damjanov et al., 1986). Recent evidence indicates that mutated EGFR may potentiate tumour invasion by upregulating genes involved in the formation and breakdown of the extracellular matrix, such as collagens and fibrillins, and matrix metalloproteinases respectively (Cox et al., 2000; Lal et al., 2002). EGFR mutations have also been linked with a good clinical response to tyrosine kinase inhibitors in NSCLC (Lynch et al., 2004; Paez et al., 2004).
Figure 5.4 Present versus absent membranous EGFR and survival

Figure 5.5 Present versus absent cytoplasmic EGFR and patient survival
High expression of EGFR has been found in a range of solid tumours including oral (Todd and Wong, 1999), gastric (Yasui et al., 1988), oesophageal (Mukaida et al., 1991), head and neck (Putti et al., 2002), transitional cell carcinoma of the bladder (Neal et al., 1985), breast (Aziz et al., 2002; Fox and Harris, 1997), pancreatic cancer and non-small cell lung cancer (Cox et al., 2000). Tumour differentiation and malignancy/invasion has been described in oesophageal (Mukaida et al., 1991), gastric (Yasui et al., 1988), head and neck squamous cell carcinoma (Putti et al., 2002), and transitional cell carcinoma of the bladder (Neal et al., 1985) although these observations are by no means consistent in all studies (Christensen, 1998).

Northern blot hybridisation has demonstrated higher levels of EGFR mRNA in colonic tumours compared with normal colonic mucosa, albeit only in 50% of the resected specimens analysed (Ito et al., 1990). Indirect immunofluorescence, western transfer and EGF binding studies by Bradley et al, demonstrated higher EGFR expression in moderate to well-differentiated carcinomas in comparison to poorly differentiated carcinomas and normal colonic tissue. Thus EGFR was suggested as a marker of tumour differentiation (Bradley et al., 1986; Hunter, 2002). Steele et al also detected a difference in EGFR expression between well and poorly differentiated tumours, but found, in contrast, that cytoplasmic detection of EGFR by immunohistochemistry in 27/30 colonic tumours showed greatest intensity in the poorly differentiated tumours. The degree of staining however varied according to the immunohistochemical visualisation technique used (Steele et al., 1990). Further works by Steele, using the more sensitive integrated optimal EGFR density method, evaluated EGFR expression in 50 patients with invasive colorectal cancer. All cases stained but some were difficult to detect by eye, which in other studies would have
been classified as negative staining. EGFR expression statistically increased with increasing tumour grade and differentiation, which may be accounted for by the increased sensitivity of the detection method employed (Steele et al., 1990).

Karamis et al studied tumour differentiation and invasion in 39 colorectal adenocarcinomas comprising 30 differentiated tumours and 9 poorly differentiated tumours. Their results revealed EGFR staining in 17 cases, in both the cell membrane and the cytoplasm, which positively correlated with serosal invasion and lymph node disease, but not differentiation, in contrast to previous works *(vide supra)* (Karameris et al., 1993).

Other studies failed to demonstrate any significant correlation between EGFR expression, tumour invasion and differentiation. Immunohistochemistry, western blotting and iodine-labelled EGF binding analysis demonstrated expression in 77.1% of 61 tumours analysed, but no statistical correlation to tumour characteristics (Yasui et al., 1988). In a larger series of 44 normal colon tissue specimens (adjacent/distant from the tumour), 25 adenomas and 144 adenocarcinomas, no clear association between EGFR expression and type of tissue was obtained, with intense staining only detected in 11 carcinoma cases. Correlation with clinicopathological parameters did not reach statistical significance, thus the authors concluded that EGFR expression in colorectal cancer is complex and may reflect individual patients’ genetic constitution (Koretz et al., 1990).

EGFR has been correlated with poor prognosis in oesophageal (Mukaida et al., 1991) and breast cancer (Aziz et al., 2002), particularly in association with high tumour
vascularity in node negative patients (Fox and Harris, 1997), although oestrogen receptor positive tumours express significantly less EGFR compared with oestrogen receptor negative tumours (Sainsbury et al., 1985). In oral cancers, the prognostic significance remains unclear (Todd and Wong, 1999). Co-expression of EGFR and CAIX was associated with a worse prognosis compared with that of either factor alone in a recent study by Swinson et al. This suggests that EGFR may enhance the expression of CAIX, act as a survival factor for cells growing in a hypoxic environment, or both (Swinson et al ASCO 2002, unpublished data).

Contrary to a large proportion of solid tumours (detailed above), the prognostic value of EGFR expression in colorectal cancer has not been widely studied.

Increased EGFR expression has been correlated to poor patient survival. In one series of 82 colorectal tumours (Mayer et al., 1992), 80/82 colorectal tumours expressed EGFR, with 51 cases displaying maximal intensity by immunohistochemical analysis. The percentage tumour staining was found to be significantly associated with worse prognosis; >50% expression was associated with shorter survival as compared with <50% expression. In a second study, elevated EGFR levels, as determined by iodine-125-labelling, were associated with poor prognosis in patients with colorectal cancers (p=0.024) and rectal cancer (p=0.002). Indeed, high EGFR was found to be independently prognostic on multivariate analysis (Reinhard et al., 2003).

In this series, which is the first to examine EGFR expression in resected colorectal liver metastases, only 34% of tumours expressed EGFR (n=62) as detected by immunohistochemistry. No significant correlations were found between EGFR and
the clinicopathological variables, which is in keeping with much of the published literature (*vide supra*) for primary tumours. No associations were found between the expression of EGFR and angiogenesis as evidenced by the lack of correlation between EGFR and MVD and VEGF. Equally, EGFR was not correlated to the expression of CAIX, in contrast to findings documented by Swinson et al in NSCLC (Swinson et al ASCO 2002, unpublished data).

Membranous EGFR in this series was statistically associated with poor patient survival (p=0.01). This suggests that EGFR both in the primary tumour, as documented by Mayer and Reinhard (Mayer et al., 1992) (Reinhard et al., 2003), and the metastatic deposits exerts an effect on patient survival.

Further investigation into the growth pathways known-to-be modulated by EGFR (*vide supra*) and possible tumour mutations may be useful. The results are in keeping with the recent observation that targeting EGFR with the therapeutic monoclonal cetuximab (C225) is an effective strategy in the management of relapsed colorectal cancer.
THE GELATINASES:

MATRIX METALLOPROTEINASE-2 AND

MATRIX METALLOPROTEINASE-9
MATRIX METALLOPROTEINASE-9

1. OPTIMISATION OF IMMUNOHISTOCHEMISTRY

The best staining was obtained by pressure-cooking the slides for 2 minutes using a 1:100 antibody dilution for MMP-9 and a dilution of 1:50 for MMP-2.

2. IMMUNOHISTOCHEMICAL EXPRESSION OF MMP-9

At microscopy at varying magnification (x40, x100, x200 and x400), two patterns of MMP-9 staining were observed: tumour cell, stromal (in fibroblasts and inflammatory cells) and peri-vascular. Tumour cell staining was the predominant pattern of staining detected (Figure 6.1). Percentage expression of MMP-9 staining was divided about the median value. These data were subsequently utilised for comparison with the clinico-pathological variables, CAIX, MVD, EGFR and survival.

MMP-9 expression was detected to a varying degree in all tumour cells: 67% (n=121) had <median MMP-9 expression and 33% (n=60) had >median MMP-9 expression. Stromal MMP-9 was detected in 70% of tumours (n=127). Of these 70%, 59.6% of cases had <median expression (including negative cases) and 29.8% had >median expression of MMP-9. The negative control; negative, +, ++ and +++ stromal MMP-9 expression are illustrated in Figures 6.2-6.6. No peri-vascular MMP-9 expression was detected.
Figure 6.1 Tumour cell MMP-9 immunoreactivity

(x100 magnification)

Figure 6.2 Negative MMP-9 immunoexpression

(x100 magnification)
Figure 6.3 Negative MMP-9 immunoreactivity in the stroma

(x100 magnification)

Figure 6.4 Stroma + MMP-9 immunoreactivity

(x100 magnification)
Figure 6.5 Stroma ++ MMP-9 immunoreactivity

(x100 magnification)

Figure 6.6 Stroma +++ MMP-9 immunoreactivity

(x100 magnification)
3. MMP-9 AND CLINICOPATHOLOGICAL DATA

Low tumour cell MMP-9 expression was associated with lesions <35mm (Table 6.1).

4. MMP-9 AND ANGIOGENESIS

Low sMMP-9 expression was significantly associated with less than the median contiguous tumour edge MVD and intra-tumour MVD (Table 6.2).

Table 6.1 Clinico-pathological variables and MMP-9

<table>
<thead>
<tr>
<th>Pathological factor</th>
<th>cMMP-9 &lt;median ≥ median</th>
<th>P value</th>
<th>sMMP-9 &lt;median ≥median</th>
<th>P value</th>
</tr>
</thead>
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</tr>
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<td>78 39</td>
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<tr>
<td>Female</td>
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<td></td>
<td>55 24</td>
<td></td>
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<tr>
<td>C</td>
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<td>59 37</td>
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<td>33 16</td>
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<td>60 25</td>
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<tr>
<td>Number of lesions</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>One lesion</td>
<td>52 23</td>
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<td>44 31</td>
<td>0.15</td>
</tr>
<tr>
<td>&gt;1 lesion</td>
<td>69 37</td>
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<td>73 33</td>
<td></td>
</tr>
<tr>
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<td>90 55</td>
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<td>51 21</td>
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<td>≥35mm</td>
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<td>83 44</td>
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</tr>
<tr>
<td>≥5cm</td>
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<td>34 20</td>
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Table 6.2 MMP-9 and MVD

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<th>sMMP-9</th>
<th>p value</th>
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<tr>
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<tr>
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<td></td>
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<td></td>
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<td>0.67</td>
<td>59</td>
</tr>
<tr>
<td>Above median</td>
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<td>23</td>
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<td>44</td>
</tr>
<tr>
<td>Contiguous edge count</td>
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<tr>
<td>Above median</td>
<td>46</td>
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<td>45</td>
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<tr>
<td>Intra-tumour hotspot</td>
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</tr>
<tr>
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<td>Above median</td>
<td>58</td>
<td>31</td>
<td></td>
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<td>Cumulative mean</td>
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</tr>
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<tr>
<td>Above median</td>
<td>50</td>
<td>28</td>
<td></td>
<td>44</td>
</tr>
</tbody>
</table>

5. RELATIONSHIP BETWEEN MMP-9, CAIX, EGFR and VEGF

There was a trend between low MMP-9 expression and low stromal CAIX expression although failing to reach statistical significance (Table 6.3).

Table 6.3 MMP-9, CAIX, EGFR and VEGF

<table>
<thead>
<tr>
<th>Mediators of angiogenesis</th>
<th>CellMMP-9</th>
<th>p value</th>
<th>sMMP-9</th>
<th>p value</th>
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<tbody>
<tr>
<td></td>
<td>&lt;median</td>
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<tr>
<td>Membranous CAIX</td>
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<tr>
<td>Below median</td>
<td>52</td>
<td>23</td>
<td>0.55</td>
<td>49</td>
</tr>
<tr>
<td>Above median</td>
<td>69</td>
<td>37</td>
<td></td>
<td>68</td>
</tr>
<tr>
<td>Cytoplasmic CAIX</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Below median</td>
<td>54</td>
<td>27</td>
<td>0.9</td>
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<tr>
<td>Above median</td>
<td>67</td>
<td>33</td>
<td></td>
<td>68</td>
</tr>
<tr>
<td>Stromal CAIX</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>38</td>
<td>0.07</td>
<td>86</td>
</tr>
<tr>
<td>Above median</td>
<td>29</td>
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</tr>
<tr>
<td>MEGFR</td>
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</tr>
<tr>
<td>Absent</td>
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<td>29</td>
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<td>89</td>
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<tr>
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<tr>
<td>Tumour cell VEGF</td>
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<td></td>
</tr>
<tr>
<td>Below median</td>
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<td>0.82</td>
<td>51</td>
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<tr>
<td>Above median</td>
<td>48</td>
<td>43</td>
<td></td>
<td>48</td>
</tr>
<tr>
<td>Stromal VEGF</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Below median</td>
<td>45</td>
<td>44</td>
<td>0.42</td>
<td>50</td>
</tr>
<tr>
<td>Above median</td>
<td>52</td>
<td>40</td>
<td></td>
<td>49</td>
</tr>
</tbody>
</table>
6. MMP-9 AND PATIENT SURVIVAL

Figure 6.7 Cytoplasmic MMP-9 and patient survival

![Graph showing cytoplasmic MMP-9 and patient survival with p=0.4.]

Figure 6.8 Stromal MMP-9 and patient survival

![Graph showing stromal MMP-9 and patient survival with p=0.07.]

1.1
1.0
C
D
0
-1000
1000
2000
3000
4000
survival from resection

-1000
0
1000
2000
3000
4000
survival from resection
7. IMMUNOHISTOCHEMICAL EXPRESSION OF MMP-2

At microscopy at varying magnification (x40, x100, x200 and x400), three patterns of MMP-2 staining were observed: tumour cell, stromal (in fibroblasts and inflammatory cells) and peri-vascular. Tumour cell staining (Figure 6.9) was the predominant pattern of staining detected. Percentage expression of MMP-2 staining was divided about the median value. These data were subsequently utilised for comparison with the clinico-pathological variables, CAIX, MVD, EGFR and survival.

Figure 6.9 Tumour cell MMP-2 immunoreactivity

(x200 magnification)
MMP-2 tumour cell expression was detected in 123 cases (68%). Including the negative cases, 90 tumours (49.7%) had <median percentage expression and 91 tumours (50.3%) had >median percentage expression. In the stroma, MMP-2 was detected in all cases, with 91 cases (50.3%) having <median percentage expression and 90 cases (49.7%) >median percentage expression. Examples of +, ++ and +++ stroma immunoreactivity are shown in Figures 6.10-6.12. Similarly, MMP-2 was detected in a peri-vascular distribution in all cases, with 96 cases (53%) expressing >median percentage expression and 85 cases (43%) <median percentage expression.

Figure 6.10 Stroma + MMP-2 immunoreactivity

(x100 magnification)
Figure 6.11 Stroma ++ MMP-2 immunoreactivity

(x100 magnification)

Figure 6.12 Stroma +++ MMP-2 immunoreactivity

(x100 magnification)
8. MMP-2 AND THE CLINICOPATHOLOGICAL DATA

MMP-2 expression did not correlate with the clinico-pathological factors (Table 6.4).

Table 6.4 Clinico-pathological variables and MMP-2

<table>
<thead>
<tr>
<th>Pathological factor</th>
<th>cMMP-2 &lt;median</th>
<th>≥median</th>
<th>P value</th>
<th>sMMP-2 &lt;median</th>
<th>≥median</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
<td></td>
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<td></td>
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<td>23</td>
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165
9. MMP-2 AND ANGIOGENESIS

A trend was observed between > median cMMP-2 and > median contiguous tumour edge vessels (Table 6.5).

10. RELATIONSHIP BETWEEN MMP-2, CAIX, EGFR and VEGF

Less than the median sMMP-2 was associated with positive tumour cell EGFR expression. sMMP-2 >median was associated with mCAIX >median (Table 6.6).

11. RELATIONSHIP BETWEEN MMP-2 AND PATIENT SURVIVAL

No statistically significant correlation was found between MMP-2 protein expression and patient survival (Figures 6.13 and 6.14).
### Table 6.5 MMP-2 and MVD

<table>
<thead>
<tr>
<th>Microvessel Density</th>
<th>cMMP-2 &lt;median</th>
<th>≥ median</th>
<th>p value</th>
<th>sMMP-2 &lt;median</th>
<th>≥ median</th>
<th>p value</th>
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<td>48</td>
<td>28</td>
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<tr>
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<td></td>
<td>37</td>
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<td></td>
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<tr>
<td>Intra-tumour hotspot</td>
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<td>41</td>
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<td>42</td>
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### Table 6.6 MMP-2 and mediators of angiogenesis

<table>
<thead>
<tr>
<th>Mediators of angiogenesis</th>
<th>CellMMP-2 &lt;median</th>
<th>≥ median</th>
<th>p value</th>
<th>sMMP-2 &lt;median</th>
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<td>Membranous CAIX</td>
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<td></td>
<td>50</td>
<td>56</td>
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<tr>
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<td></td>
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<tr>
<td>Stromal CAIX</td>
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<tr>
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<td>41</td>
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Figure 6.13 Tumour cell MMP-2 and patient survival

Figure 6.14 Stromal MMP-2 and patient survival
12. DISCUSSION

Degradation of the extracellular matrix and the basement membrane is facilitated by the up-regulation of enzymes known as matrix metalloproteinases (Cox et al., 1999). These enzymes are secreted as pro-enzymes by fibroblasts, stromal and endothelial cells, and are subsequently activated by conformational changes (Chambers and Matrisian, 1997).

The gelatinases, or MMP-2 and MMP-9, have been isolated in a variety of solid tumours, both in the stroma and in the tumour cell. High levels of metalloproteinases have been located to the invasive tumour edge. In primary colorectal cancer, most studies appear to agree that increased levels of the gelatinases are found in carcinomas as compared with adenomas, although not all studies have found the presence of gelatinases in adenomas.

Latent and active forms of MMP-9 have been found in colorectal liver metastases, particularly at the invading tumour edge. Interestingly, however, higher levels of latent gelatinase forms have been associated with increased risk of disease recurrence, and low levels of MMP-2 with larger metastases.

In this series, extensive MMP expression was detected. In 95.5% of cases, greater than 50% of tumour cells expressed MMP-9, with 85% of cases expressing MMP-9 in 70% or more tumour cells. MMP-2 was also widely expressed albeit not as extensively as MMP-9, with 48% of tumours expressing >50% tumour cell MMP-2, and 38% of tumours expressing 70% or more tumour cell MMP-2. Similarly,
extensive expression was detected in the stroma. The distribution of tumour MMP expression, however, was not predominantly at the tumour edge, in contrast to findings published by Zeng’s group (Zeng and Guillem, 1995).

A number of findings have been seen in this study regarding the relationship between MMP-2 and -9 and clinicopathological variables, MVD, CAIX and EGFR.

Statistically less tumour cell MMP-9 was associated with smaller lesions (<35mm), and below the median stromal CAIX expression. Less than the median stromal MMP-9 expression was significantly associated with less than the median contiguous tumour edge MVD and intra-tumour MVD, and although not statistically significant, with less than the median tumour edge hotspots. These findings seem to suggest that decreased expression of MMP-9 is associated with decreased tumour invasion, reflected by the smaller lesions and reduced vascularity, particularly at the tumour edge. Previous work has demonstrated that the edge of the colorectal liver metastasis is biologically important since high vascularity (angiogenesis) at this site is an independent negative prognostic factor. Although not statistically significant, a trend was observed towards poor prognosis with higher levels of MMP-9 expression.

In contrast, MMP-2 did not correlate with any of the clinico-pathological variables, and very interestingly, less than the median tumour cell MMP-2 was associated with less than the median contiguous tumour edge vessels, although not statistically significant. MMP-2 < median was associated with positive tumour cell EGFR expression, and sMMP-2 > median was associated with greater than the median
mCAIX. There was no correlation with VEGF expression. No association with patient survival was observed.

These findings may suggest that MMP-9 has an important role in facilitating tumour invasion in colorectal liver metastases by promoting angiogenesis. MMP-9 may thus be associated with more aggressive tumours, in keeping with Zeng's observations (Zeng and Guillem, 1998), wherein the invading tumour edge contained increased levels of both mRNA and protein expression of MMP-9. Interestingly, <median MMP-2 was associated with < median contiguous vasculature at the edge of the tumour (although failing to reach statistical significance). Stromal MMP-2 expression did correlate with membranous CAIX expression. This may suggest a relationship between the CAIX driven micro-environmental acidity and MMP-2 expression: as discussed above MMP-2 appears to be more pH sensitive than MMP-9 (Razaq et al., 2003) which may explain why expression of MMP-9 does not appear to be related to CAIX expression. It would be interesting to evaluate tumour pH in relation to CAIX and MMP expression to further investigate this theory.
HYPOXIA INDUCIBLE FACTOR -1
1. STAGE 1

The first aim was to reproduce the method that worked using sections of NSCLC (unpublished data) with known positive controls (see below).

Frozen, aliquoted samples of HIF-1α67 primary antibody which were used to stain the locally compiled database of NSCLC were utilised with a CSA kit (DAKO, Ely, UK) well within the expiry date.

Two different control tissue types, used by the previous researcher in NSCLC, thus expected to yield high expression of HIF-1α and corresponding negative controls (Courtesy of Dr D Swinson, Research Registrar in Oncology) were used.

Tumour sections highly expressive of nuclear HIF-1α were selected from the NSCLC database. Fresh sections were cut using the microtome. Three different control sections of clear cell renal carcinoma were identified and fresh sections cut.

Sections of liver metastases highly expressive of CAIX were selected from the current study database, based on the assumption, as ascertained from the literature, that CAIX is up-regulated by HIF-1α. These sections would therefore be expected to yield HIF-1α expression.
2. STAGE 2

Using the CSA method described (*vide supra*), which was the exact method used to detect HIF-1α in NSCLC, was followed. Primary antibody concentrations of 1:100, 1:500 and 1:1000 were used on sections from NSCLC, renal cell carcinoma and liver metastases. Each concentration was incubated for either one hour or overnight, as per protocol. No detectable staining was achieved. This method was replicated by a fellow investigator to identify possible methodological errors by the principal researcher. No detectable staining was yielded.

3. STAGE 3

A new positive control slide had to be selected from the NSCLC database as no further sections could be obtained from the first NSCLC tumour sample used. To achieve this, the 10 most highly expressive cases of HIF-1α documented in the database were selected, and the corresponding H&E slides (obtained from Histopathology) reviewed to ensure that sufficient tumour cells were present. The paraffin-embedded tissue blocks were subsequently examined in order to select those with the maximum quantity of embedded tumour for sectioning. The CSA method, now confirmed to be as per the original protocol, was subsequently followed without success.
4. STAGE 4

In order to evaluate the possibilities of rapid kit deterioration, loss of tissue antigenicity, or loss of antibody activity, a new CSA kit, used within a day of receipt, a newly-purchased primary antibody and freshly cut tissue sections were employed. All solutions were made-up *de novo* with precise pH measurements for each experiment. Once again, no detectable staining was achieved, following the accepted protocol.

The literature was reviewed to identify any other possible methods, and interestingly, success was reported with own-laboratory primed antibodies, but subsequent reports did use the same HIF-1α67 antibody. Some groups also experienced difficulties in optimising the methods, as per e-mails directed to the NSCLC researchers.

5. STAGE 5

Since the Envision visualisation system (Envision, DAKO, Ely, UK) had yielded very good results when used with CAIX, p53, MVD, CAIX and the MMPs, and did not require overnight incubation, the method was attempted with the HIF-1α67 antibody. No success.

6. STAGE 6

Having not yielded any significant tissue section staining with the above methods described, other factors affecting antibody behaviour were considered. Of note was
the temperature in the laboratory. All the optimisation experiments hitherto had been undertaken in an air-conditioned laboratory, with temperatures recorded at 20\(^\circ\)C.

Former experiments had been undertaken in an adjacent laboratory without air conditioning. Thus, experiments using the original CSA protocol were carried out in said laboratory, with fresh sections and a new kit.

The NSCLC and renal cell controls were utilised with the CSA system, which appeared to yield some nuclear staining as expected.

The experiment was repeated with liver sections, but excessive background staining was obtained. A biotin blocking system (CSA Ancillary System, DAKO, Ely, UK) was used (see methods) in order to reduce non-specific background staining. However, despite repeated experiments with the liver tissue, no clear, HIF-1\(\alpha\) specific nuclear staining was detectable. There was excessive background staining despite the biotin-blocking system, and upon consultation with two Consultant Histopathologists, the staining was deemed uninterpretable.

7. SUGGESTIONS FOR IMPROVEMENT

Unfortunately, despite considerable attempts to detect HIF-1\(\alpha\) protein expression by immunohistochemistry in resected colorectal liver metastases, no interpretable results were yielded.
The first problem was achieving any form of staining by using the only commercially available antibody to HIF-1α. The antibody is clearly very sensitive to environmental factors, as evidenced by positive expression being yielded by working in an air-conditioned laboratory as compared with working at room temperature. Although this issue appeared to be finally addressed, a more resilient antibody may be more helpful, especially if reliable, reproducible results are to be documented.

The most significant problem that ultimately prevented the expression of HIF-1α from being evaluated in this series of colorectal liver metastases was the inability to achieve tumour-specific staining. Much background staining appeared on the slides, rendering any specific HIF-1α staining uninterpretable. This probably arose secondary to the biotin-based development system, which was the only method available to immunohistochemically detect HIF-1α with the antibody available. Once again, a novel antibody that could be detected by using a non-biotin-based system may address this issue.
CONCLUSIONS AND FUTURE WORK
1. CONCLUSIONS

In this study, growth factor signalling is represented by the epidermal growth factor receptor; hypoxia is represented by CAIX and necrosis; angiogenesis by microvessel density and vascular endothelial growth factor and extracellular remodelling by the matrix metalloproteinases MMP-2 and MMP-9. The most important findings are stated below, followed by a hypothesis of the inter-relationship between these factors in resected colorectal liver metastases.

1) This is the largest series to date to evaluate the expression of CAIX, EGFR, MMP-2 and MMP-9 plus the already established MVD and VEGF in resected colorectal liver metastases.

2) Membranous CAIX and membranous EGFR are significantly associated with poor overall patient survival.

3) Tumour necrosis is significantly associated with poor patient survival, but does not correlate with any of the factors evaluated.

4) MMP-9 is significantly associated with increased tumour edge contiguous vessels and central tumour vessels, suggesting a role in angiogenesis and tumour invasion.

5) MMP-2 is associated with CAIX expression that may be explained by the role of CAIX in acidification, and the importance of such acidification in the regulation of metalloproteinase activity.

6) Membranous CAIX, membranous EGFR and tumour necrosis are independently negative prognostic factors in colorectal liver metastases using the Cox Regressional Model (p=0.019, p=<0.001 and p=0.027 respectively).
2. THE BEHAVIOUR OF COLORECTAL LIVER METASTASES IN RESPONSE TO HYPOXIA: A THEORY

The following hypothesis is proposed:

Protein expression of CAIX and VEGF in response to hypoxia (as documented in the literature) may generate an acid extracellular environment and increased vessel permeability (on the assumption that they are working in the same way as previous experimental data suggests). The extracellular acidity which, based on the mechanisms by which metalloproteinases function must be present, may up-regulate matrix metalloproteinases (MMP-2 correlation with CAIX) leading to extracellular remodelling to sustain developing blood vessels (significant correlation between MMP-9 and tumour edge vessels and central tumour vessels) and consequent tumour invasion (experiment performed on metastatic deposits, with sizes ranging from 4mm to 230mm, median size 35mm).

Naturally, only a minute understanding of the biology of colorectal liver metastases has been yielded thus far. However, interesting findings were noted which, if applied to the diagnostic pathway, may help identify patients with worse prognosis and in the future, facilitate targeted chemotherapy.

Although no correlation was demonstrated between EGFR and the potential markers of hypoxia, necrosis, CAIX, VEGF evaluated in this study, the growth factor has an adverse effect on prognosis and has, in both experimental and clinical studies been shown to be a potential survival factor for tumour cells within a hypoxic environment.
3. FUTURE WORK

3.1 Completion of immunohistochemistry

In order to complete the immunohistochemical analysis of growth factors relating to hypoxia and angiogenesis in this series, protein expression of HIF-1α would need to be examined. Therefore, the first aim would be to investigate novel immunohistochemical techniques to detect the protein expression of HIF-1 alpha, using more effective antibodies (which are becoming available) and non-biotin based visualisation systems.

Secondly, the series could be expanded to include primary tumours and their corresponding metastatic deposit. This would enable comparisons to be made between the expression of growth factors in both colonic primary tumours and the liver metastases. This was initially attempted, but numerous constraints prevented a full series to be obtained, principally due to the tertiary referral status of many patients.

Further confirmation of the results obtained could be achieved by repeating all the experiments in a larger, prospective series.

3.2 Further experimental techniques

Having investigated the immunohistochemical expression of microvessles, VEGF, thrombospondin-1, p53, CAIX, EGFR, MMP-2 and MMP-9 in this series of
colorectal liver metastases, further assessment of these growth factors could be undertaken by using different, \textit{in vitro} and \textit{in vivo} experimental techniques

\textbf{3.1.1 \textit{In vitro} work}

Gene expression of these growth factors using tissue from appropriately consented patients obtained during liver resection could be studied. After yielding viable cells through tissue culture techniques, locally available methods such as western blotting, ELISA and PCR could be optimised in order to further this work. Characterisation of the EGF receptor would be of interest.

Changes in gene expression and cell behaviour in response to different environmental conditions could be investigated. Of particular interest would be to examine potential changes in gene expression in relation to different levels of oxygenation, by exposing colorectal cancer cells to hypoxia, using a commercially and locally available hypoxic chamber. It may be hypothesized that hypoxic conditions would up-regulate the expression of certain growth factors such as VEGF.

The effects of chemotherapy and resection techniques both on patient survival and on the expression of hypoxia and angiogenesis-related growth factors could be considered. The presently ongoing European Organisation for the Research and Treatment of Cancer trial is comparing outcomes in patients having either liver resections or ablative techniques plus or minus pre-operative chemotherapy. Comparing the presence of growth factors in patients having undergone pre-liver resection chemotherapy with those who have not may yield interesting results.
3.1.2 In vivo work:

The next area of investigation would concern assessing tumour behaviour in vivo. Assessing the effects of tumour hypoxia could be achieved by measuring oxygen concentrations in the tumour intra-operatively, and by subsequently correlating tissue hypoxia at surgery with gene and protein expression in fresh and paraffin-embedded tissues, as already described in cervical cancer.

Angiogenesis and tumour necrosis could be evaluated by physiological studies using 3D CT or MR reconstruction to investigate tumour vascularity and necrosis.

4. POTENTIAL IMPACT ON PATIENT CARE

The identification of negatively prognostic factors in colorectal liver metastases may aid in the development of specific anti-factor antibodies, such as the anti-angiogenic/anti-hypoxia agents already described. The ultimate aim would therefore be to facilitate the development of tumour specific, targeted chemotherapy.

Routine immunohistochemical analysis of the growth factors found to be negatively prognostic could be implemented, to identify those patients at high risk of poor survival and who may benefit from a targeted chemotherapeutic regime. The techniques and visualisation systems used are already in routine use in Histopathology laboratories and should not incur further significant cost.
REFERENCE LIST


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PUBLISHED ABSTRACTS

Carbonic anhydrase IX independently predicts survival in colorectal liver metastases
Marshall LJ, Sutton CD, Jones LJ, O'Byrne K, Dennison AR, Poston G, Lloyd DM, Berry DP
Br J Surg 2003; 90: Supp 1, 51
Association of Surgeons of Great Britain and Northern Ireland, Manchester, May 2003

Biological factors influencing angiogenesis and prognosis in resected colorectal liver metastases
Association of Surgeons of Great Britain and Northern Ireland, Harrogate, April 2004

POSTERS

Carbonic anhydrase IX independently predicts for poor prognosis in patients with colorectal liver metastases
Leicestershire Research Prize Day, 2002

Hypoxia and angiogenesis in colorectal liver metastases
Association of Surgeons of Great Britain and Northern Ireland, Glasgow, April 2005
Br J Surg; 92, Supp 1: 58

PAPERS IN PROGRESS

Carbonic anhydrase IX independently predicts for poor prognosis in patients with colorectal liver metastases.

Epidermal growth factor receptor independently predicts for poor prognosis in patients with colorectal liver metastases.