Hypoxic Markers in Non-Small Cell Lung Cancer

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by

Dr. Daniel Swinson

University of Leicester

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ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

ABSTRACT

Hypoxic markers in Non-small cell lung cancer. D.E. B. Swinson

Hypoxia is an important factor in the pathogenesis of solid tumours. Hypoxia inducible factor (HIF)-1α and HIF-2α are transcription factors that in part mediate the cellular response to hypoxia. These transcription factors are involved in the regulation of angiogenesis, anaerobic metabolism, pH homeostasis, erythropoiesis and cell death.

Immunohistochemical (IHC) assays were optimised for HIF-1α and one of its transcriptional targets, Carbonic Anhydrase (CA) IX. Attempts to optimise an IHC assay for HIF-2α failed to produce reproducible staining. A scoring system was also devised to assess the extent of tumour necrosis (TN) in tumour sections. The expression of these factors was assessed in a retrospective series of patients who had NSCLC tumours resected with curative intent. The expression of EGFR, p53, Bcl-2, MMP-2 and MMP-9 and angiogenesis had previously been assessed.

Extensive TN, perinuclear (p) CA IX and high HIF-1α expression were associated with a poor prognosis. PCA IX, stage, gender, MMP-9 and angiogenesis were independent prognostic factors.

The spatial relationship between membranous CA IX expression and TN and tumour microvessels support other studies proposing that CA IX is a marker of tumour hypoxia.

EGFR expression was associated with pCA IX, membranous (m)CA IX and HIF-1α expression. *In vitro* studies demonstrated that prolonged treatment with the EGFR tyrosine kinase inhibitor, ZD1839 suppressed CA IX expression. These results suggest that activated EGFR may induce CA IX. As such co-expression of these factors may identify patients that are more likely to respond to EGFR targeted therapies.

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Activated microvessel count	aMVC	Microvessel density	MVD
Adenosine triphosphate	ATP	Nip 3 like protein X	NIX
Aryl hydrocarbon nuclear translocator	ARNT	Nitric oxide	NO
American society of clinical oncology	ASCO	Non-steroidal anti-inflammatory drug	NSAID
Basic fibroblastic growth factor	bFGF,	Non-small cell lung cancer	NSCLC
Basic-helix-loop-helix	bHLH	Oxygen	O_2
Bcl-2/adenovirus EIB 19kD-interacting protein 3	BNIP3	Pressure cooking	P/c
Cytoplasmic	c	Perinuclear	p
Carbonic anhydrase	CA	Type-1 plasminogen activator inhibitor-1	PAI-1
Cell mediated immune response	CMI	Platelet derived endothelial cell growth factor	PDECGF
Continuous hyper fractionated accelerated radiotherapy	CHART	Partial pressure of oxygen	pO_2
Chronic obstructive pulmonary disease	COPD	Protein kinase C	PKC
Cyclooxygenase	COX	Intracellular pH	pHi
Diaminobenzidine tetrahydrochloride	DAB	Pleckstrum homology	PH
Ductal carcinoma in situ	DCIS	Phosphatidylinositol 3 kinase	PI3K
Diffusion limited fraction	DLF	Phosphatase and tensin homolog deleted on chromosome ten	PTEN
Dimethyl sulphoxide	DMSO	SH2-domain-containing inositol phosphatase	SHIP
Dominant negative	DN	Sodium dodecylsuphate	SDS
Ethlyenediaminetetraacetic acid	EDTA	SDS polyacrylamide gel electrophoresis	SDS-PAGE
Epidermal growth factor receptor	EGFR	Smooth muscle actin	SMA
Hepatocyte growth factor	HGF	Tris buffered saline	TBS
Heparin binding growth associated molecule	HB-GAM	tetramethylenediamine	TEMED
Humoral immune response	HI	Tris/glycine	T/G
Hypoxic inducible factor	HIF	Tumour-associated macrophages	TAM
Hypoxia response element	HRE	Tissue factor	TF
Horseradish peroxidases	HRP	transforming growth factor	TGF
Immunohistochemistry	IHC	Thymus helper cells	Th cells
Interstitial fluid pressure	IFP	Trypsin/ EDTA	T/E
Insulin-like growth factor-1 receptor	IGF-1R	Tyrosine kinase inhibitor	TKI
Interleukin	IL	Thrombospondin	TSP
Industrial methylated spirit	IMS	Tumour necrosis	TN
Ischaemic heart disease	IHD	Tumour necrosis factor	TNF
Low power field	LPF	Urokinase-type plasminogen activator	uPA
Mitogen associated protein kinase	MAPK	uPA receptor	uPAR
Membranous	m	Vascular endothelial growth factor	VEGF
Mitomycin c, ifosfamide and cisplatin	MIC	VEGF receptor	VEGFR
Matrix metalloproteinase	MMP	Von Hippel-Lindau	VHL
Mean vascular pressure	MVP		
Mitomycin c, vinblastine and cisplatin	MVP		

Chapter 1

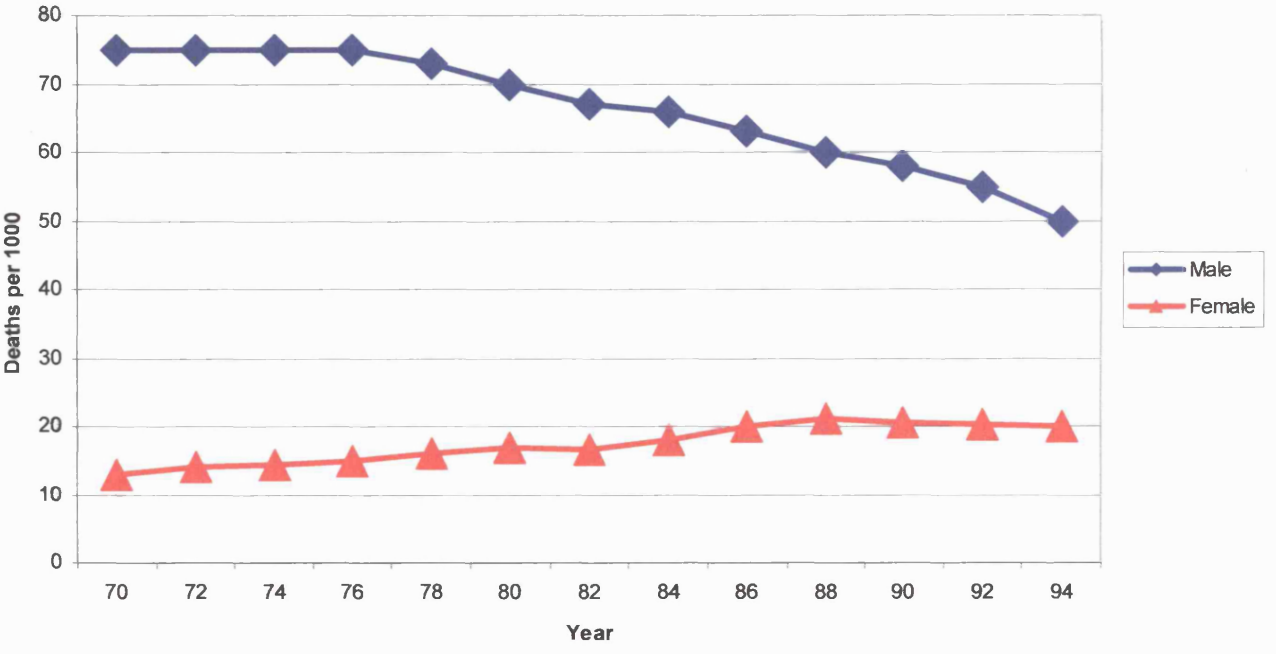
Introduction

1.1. LUNG CANCER: BACKGROUND AND CURRENT TREATMENTS

1.1.1. Epidemiology

Lung cancer is the commonest cancer in the world and the third most common cause of death in the UK. The highest incidence rates are seen in Europe and North America. In the year 2000 an estimated 375 000 patients were diagnosed with lung cancer, of which the resultant mortality was 347 000 deaths. The incidence is 4 fold higher in men than women. This gender differential has been changing since the 1970s as the incidence has been falling in men but increasing in women in Europe and North America (figure 1) [1-3].

Figure 1.1. Northern European lung cancer incidence rates in men and women from 1970-1994 (age-standardised rates, world standard population)



The incidence of lung cancer has been falling for the last 30 years but the survival of the patient faced with the diagnosis of lung cancer has remained relatively static although there has been a slow upward trend. The reported 5-year survival in 1960 for resectable Non-small cell lung cancer (NSCLC) was 23% and in 1990 it was 54%. This improvement has been attributed to improvements in pre-operative patient selection rather than improved medical intervention [4]. The median of median survivals for NSCLC patients participating in clinical trials in North America from 1973 to 1985 was 9.1 months compared to 11 months from 1986-1998 [5].

1.1.2. Environmental and genetic causes

1.1.2.1. Smoking

Over the last 30 years the incidence of smoking has fallen in the male population but increased in the female population mirroring the incidences of lung cancer. This trend is particularly marked in adolescents. A report released by the WHO this year based on over 1 million adolescents from 150 countries found that young girls are now smoking almost as much as boys. The male to female smoking ratios were consistently smaller among youth than among adults (table 1.1)[6].

Table 1.1. Male to female smoking ratios by WHO region [6]

WHO region	Adult	Adolescent
Africa	7.1:1	2.2:1
Americas	1.6:1	1.2:1
Eastern Mediterranean	8.8:1	4.3:1
Europe	1.8:1	1.2:1
South East Asia	11.0:1	4.2:1
Western Pacific	7.5:1	1.7:1

The WHO currently attributes 4.9 million deaths per year to tobacco use. If the increase in adolescent female smoking is translated to adulthood this attrition rate could double over the next 2 decades [6].

Cigarette smoking is responsible for 80% of cases of lung cancer. Doll and Hill's seminal paper in 1950 based on a 600 patient case control series reported a 20-30 fold increase in the lifetime risk of developing lung cancer for the persistent smoker [7]. In 1994 a 40 year follow up of 40 000 doctors registered in Britain confirmed the findings of the initial Doll and Hill study [8]. A dose response relationship between the number of cigarettes smoked per day and the mortality per 100 000 men was reported, which was more marked than either that for ischaemic heart disease (IHD) or chronic obstructive pulmonary disease (COPD) (table 1.2.).

Table 1.2. Annual mortality per 100 000 men by smoking habits [8]

Smoking habit	IHD	COPD	Lung cancer
≥25/ day	1025	225	355
15-24/ day	892	112	208
5-14/ day	802	86	105
Non-smoker	572	10	14

1.1.2.2. Other environmental factors

Other environmental risk factors include asbestos, uranium, radon and certain types of nickel exposures [9, 10]. These less common environmental factors may be more important to the smoker than the non-smoker as synergy has been reported between some of these factors and tobacco [11, 12]. For example over 80% of deaths attributed to radon occur in smokers or ex-smokers [10].

1.1.2.3. Familial risk factors

Environmental factors are not the sole determinants for the risk of developing lung cancer. Only ~15% of people that have smoked ≥25 cigarettes a day for 40 years will die of lung cancer [8]. Case control studies examining the risks of developing lung cancer in family members of lung cancer patients report a 2-fold increased risk [13].

Several studies have identified candidate genes that may confer an increased risk of developing lung cancer. Potential carcinogens often require activation before they can induce malignant transformation. The rate of either activation or detoxification of carcinogens can therefore impact on the susceptibility to lung cancer of any one individual, independent of the degree of exposure to environmental factors. Examples of activating genes are those coding for CYP1A1 (aryl hydrocarbon hydroxylase) and CYP2D6, which are cytochrome P450 enzymes [14]. An example of an enzyme involved in detoxification is Glutathione S-Transferase [15]. A high rate of polymorphisms within the genes coding these enzymes has been reported in lung cancer patients [14-22].

In addition to genes involved in cellular metabolism, mutation of genes involved in regulation of the cell cycle such as the retinoblastoma (Rb) or the p53 gene impart an increased risk of developing a variety of cancers, of which lung cancer is one [23, 24].

Although genetic phenotypes appear to exist that confer an increased risk of developing lung cancer, the degree of inconsistency in studies reported precludes use of phenotype profiling in any screening program [15, 25]. Studies using larger cohorts of lung cancer patients are attempting to identify high risk genetic mutations that may be used in a screening program [26].

1.1.3. Histological Classification

Lung cancer can be divided into 2 main categories, small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), the latter accounting for 80% of cases [27]. NSCLC is a generic term that encompasses 3 histological subtypes, adenocarcinoma, squamous cell and large cell carcinoma. In the USA squamous cell subtype was the most common in the middle of the last century but this accolade now belongs to adenocarcinoma. Reasons for this are unclear but in part it has been attributed to changes in smoking behaviour and cigarette design [28].

1.1.4. Current treatments

Historically the options for the treatment of NSCLC were surgery or radical radiotherapy for early and locally advanced disease (stage I-III) or best supportive care (BSC) for advanced disease (inoperable stage III not suitable for radical radiotherapy and stage IV). BSC is a term that covers the management of symptoms such as pain and breathlessness with palliative radiotherapy and support from a varied team of healthcare workers. The use of chemotherapy remained a contentious issue until the end of the 20th century.

1.1.4.1. The use of chemotherapy in NSCLC

A meta-analysis of 52 clinical trials of platinum based chemotherapy in NSCLC provided firm evidence for the use of chemotherapy in addition to BSC in selected physically fit patients with either inoperable stage III or stage IV disease [29]. A moderate improvement in life expectancy without a negative impact on quality of life was observed. These findings have been subsequently supported by a series of studies comparing chemotherapy plus BSC with BSC alone [30-34].

The meta-analysis and preliminary studies used older chemotherapeutic agents, such as vincristine, etoposide, ifosfamide and mitomycin C in conjunction with cisplatin. A number of new

agents have been studied in clinical trials either as single agents or paired with either cisplatin or carboplatin. These drugs include gemcitabine, paclitaxel, docetaxel and vinorelbine. Studies have reported little difference between these drugs in terms of quality of life and survival [35-38]. Furthermore recent studies have not consistently found improved results when comparing the new drugs in combination with platinums with the older drugs combined with cisplatin. Rudd *et al.* reported a significant improvement in life expectancy in favour of gemcitabine plus carboplatin compared to mitomycin c, ifosfamide and cisplatin (MIC), in the absence of a superior response rate [39]. Two similar trials have been completed following Rudd *et al.*'s study that have combined either gemcitabine or docetaxel with carboplatin and compared them to MIC or mitomycin c, vinblastine and cisplatin (MVP). No difference has been reported in terms of survival but the docetaxel combination was associated with an increase in myelosuppression, infection, mucositis and alopecia [40, 41]. An older study comparing gemcitabine and cisplatin with MVP also reported no survival difference [42]. There has been a trend towards replacing cisplatin with carboplatin on the premise that carboplatin has equivalent efficacy but less toxicity. However studies comparing these 2 drugs directly have consistently reported a superior response rate and when adequately powered, reported survival advantages for cisplatin-based chemotherapy [43-46]. Gemcitabine and carboplatin is now the standard regime in the UK and is being used as a reference arm for phase III trials of new biological agents. The controversy concerning carboplatin and cisplatin could be resolved if an adequately powered, correctly dosed phase III trial comparing gemcitabine and cisplatin with gemcitabine and carboplatin was performed.

Unresectable stage III patients and stage I-III patients who are not fit for surgery are currently treated with sequential chemo-radiotherapy. The importance of both the radiotherapy and the chemotherapy components has been evaluated in clinical trials. Omission of either results in decreased local and distant control and reduced overall survival [29, 47-50]. Further improvements

in long term survival have been achieved by administering the chemotherapy concurrently with the radiotherapy but at the cost of increased toxicity [51, 52]. A further approach to improving the efficacy of radiotherapy is to increase the frequency of treatments with the aim of overcoming tumour repopulation between treatments. Conventional radical radiotherapy consists of once daily treatments where as continuous hyper fractionated accelerated radiotherapy (CHART) delivers 3 treatments a day. The latter approach has been shown to be superior to conventional radiotherapy but due to logistical problems has not been instituted widely in the UK [53]. The combination of CHART concurrently with chemotherapy has not resulted in increased survival compared to sequential chemo-radiotherapy, possibly due to increased toxicity. Induction chemotherapy prior to CHART seems to be more effective than sequential chemo-radiotherapy but unfortunately a recent trial was closed early and a significant difference was not shown [54].

A number of studies have investigated the use of adjuvant chemotherapy in NSCLC. The 1995 meta-analysis reported a non-significant 5% survival improvement at 5 years ($p=0.08$) in favour of adjuvant chemotherapy. The most commonly used combinations were cisplatin based. Since then three trials have been completed. The first trial compared adjuvant chemotherapy and radiotherapy with adjuvant radiotherapy alone in stage III NSCLC. No survival difference was found ($p=0.7$) [55]. The ALPI trial compared adjuvant chemotherapy to no chemotherapy. Although at 5 years the chemotherapy arm had a superior survival there was increased early mortality and the survival differences were non-significant [56]. The IALT trial was the largest and compared adjuvant chemotherapy to no chemotherapy. A significant survival advantage was reported at 5 years of 4% in the treatment arm ($p=0.03$) [57]. Taken as a whole these studies suggest that adjuvant chemotherapy does have a role and the addition of post operative radiotherapy may be detrimental. Taking into account the negative result of the ALPI, careful patient selection is required prior to adjuvant chemotherapy to ensure early mortality is avoided.

The role of neoadjuvant chemotherapy in early and, or locally advanced NSCLC in conjunction with surgery is being assessed in a number of trials run by the EORTC and MRC [58].

1.1.4.2. The advent of biological therapy

In light of the definite but limited impact of chemotherapy on patient survival, drug development has undergone a change in direction. Novel "biological" targeted agents have been developed that are designed to inhibit tumour growth survival factors and are undergoing clinical trials as single agents or in combination with cytotoxics [59-63]. These targets include the epidermal growth factor receptor (EGFR), vascular endothelial growth factor (VEGF) and members of the matrix metalloproteinase (MMP) family. The biological activities of these tumour factors include stimulation of angiogenesis, tumour invasion and, or increased tumour cell proliferation and, or inhibition of apoptosis, which will be discussed later.

The most promising results have emanated from studies of EGFR targeted monoclonal antibodies and EGFR selective tyrosine kinase inhibitors (TKI). EGFR is a transmembrane receptor tyrosine kinase that has been identified as an important growth factor in NSCLC [64]. The most extensively studied agent is ZD1839 (trade name Iressa), which is an EGFR TKI. Two phase II studies, IDEAL 1 and 2 involving over 400 heavily pre-treated patients, reported response rates that ranged from 8.8-18% and higher rates of symptom control. The median survival ranged from 6-8 months in a group of patients with an expected median survival of 2-3 months [65-67]. On the basis of these 2 studies the drug has been granted a licence in Japan and has been approved by the Federal Drug Association in the USA. In view of these promising results it was hoped that the addition of ZD1839 might improve the response to first line chemotherapy. The INTACT studies explored this question and 2130 patients were accrued worldwide. There were 2 treatment arms with different doses of ZD1839 and a placebo arm. All patients received concurrent standard chemotherapy.

Disappointingly, no significant difference was found between the treatment arms and the placebo arm in terms of either survival or quality of life [68, 69]. The lack of an additive effect could have been due to ZD1839 inhibiting cell proliferation thereby inducing a protective effect against the cytotoxic agents. Such a phenomenon has been seen with Tamoxifen in breast cancer when used concurrently with chemotherapy [70, 71]. The temporal relationship between the administration of ZD1839 and chemotherapy may have an important influence on the resultant anti-tumour effect and future studies exploring whether delaying administration of ZD 1839 could improve efficacy are required.

Other EGFR targeted treatments have shown promise in smaller trials. OSI 774 trade name "Tarceva" is an EGFR TKI and in a phase II study of 56 patients with advanced EGFR positive NSCLC an 11% response rate was observed [62]. Phase II studies combining anti-EGFR monoclonal antibody (IMC-C225) with chemotherapy have not reported higher than expected response rates apart from a study in the 2^{nd} line setting, but the numbers are very small and larger phase III studies are required [72-75].

VEGF is a potent angiogenic growth and vascular permeability factor, the function of which will be discussed later [76]. A monoclonal antibody (rhuMAb VEGF) targeting the VEGF has been reported to increase response rate by 10% compared to chemotherapy alone in a randomised phase II study. Disturbingly, a high incidence of severe haemoptysis was observed associated with central squamous carcinomas. A phase III trial is now under way excluding this high-risk patient group [77]. VEGF receptor TKIs have also been developed including, SU5416 and ZD6474. The safety and efficacy of SU5416 used as a single agent at low doses have been reported in Kaposi's sarcoma [78]. Trials combining EGFR and VEGF targeted treatments are under way [79].

MMPs are a family of zinc dependent neutral endopeptidases that are collectively capable of degrading all components of the extracellular matrix and have been implicated in angiogenesis and tumour invasion [64]. A non-selective MMP inhibitor, Marimastat and a relatively selective inhibitor, Prinomastat, have been developed. Phase III studies combining Marimastat in SCLC and prinomastat in NSCLC sequentially with chemotherapy have been negative [80, 81]. A further phase III study of Marimastat administered after chemotherapy, radiotherapy or surgery in stage III NSCLC patients has reached accrual and results are awaited [82].

The development of markers to identify sensitive patients likely to respond to targeted therapies may improve the response rates and be more cost effective. This has been possible with hormone therapies and Herceptin treatment in breast cancer. Unfortunately the level of EGFR membrane expression determined by immunohistochemistry does not usefully predict response to EGFR targeted therapies [83]. Studies trawling through phase II and phase III data have reported some subgroups that have higher response rates such as bronchiolalveolar carcinoma, adenocarcinoma with bilateral diffuse lesions, a high CEA and patients that have a rash as a side effect [84-87]. Ongoing studies in animal models and tumour tissue are identifying genes associated with response to ZD1839. Preliminary results are too premature to indicate whether these methods will be of use [88, 89].

The design and development of targeted therapy remains in its infancy. To date the results are promising with agents such as ZD1839 and rhuMAb indicating real clinical benefit, all be it in the setting of phase II clinical trials. An improved understanding of tumour biology will aid drug design and enable clinicians to target the use of these drugs more accurately with regard to patient selection and timing of treatment.

1.2. TUMOUR HYPOXIA

For tumours to form, grow and metastasise they need to acquire the capacity to avoid host immune surveillance, prevent programmed cell death and develop an independent blood supply from the existing vasculature, a process known as angiogenesis. Counter-intuitively a microenvironment that is lacking oxygen (O_2), i.e. hypoxic, has an important role to play in initiating and maintaining these requirements [90-92].

Tumour cell hypoxia has long been recognized to be associated with resistance to radiotherapy and a poor outcome in NSCLC. G. Schwarz in Vienna first described the O_2 dependency of irradiation in 1909. In 1953 Gray *et al.* demonstrated that the absence of O_2 at the time of irradiation reduced tumour cell sensitivity to treatment [93, 94]. Radiation produces free radicals that damage tumour cell DNA initiating apoptosis. These lesions require the presence of O_2 for permanency as under hypoxic conditions they are repaired by the donation of $H^{\bullet}$ from sulphydryl groups [95]. More recently direct measurement of the intratumoural pO_2 using polarographic needle electrodes have shown that hypoxia induces resistance to radiotherapy in cancers of the uterine cervix and head and neck and soft tissue sarcomas (reviewed by Hockel *et al.* [96]).

Importantly in the cervical carcinoma studies, patients treated with radical surgery were included in addition to those treated with radiotherapy and the disadvantage conferred by hypoxia was found to be independent of the mode of treatment. This finding indicates the malevolent effect of tumour hypoxia is not restricted to reducing sensitivity to radiotherapy but has a more general role in the pathogenesis of malignant disease. With regard to other modes of anti-cancer treatment, hypoxia has been shown to increase resistance to endocrine agents and to some forms of chemotherapy both *in vitro* and *in vivo* [97-99]. There are several reasons why a hypoxic tumour may be relatively protected from the effects of chemotherapy, which are listed in table 1.3.

Table 1.3. Mechanisms by which hypoxia may reduce sensitivity to chemotherapy

1	Reduced blood supply will limit drug delivery [100].
2	Increased mutational rate will select for apoptotic resistant clones [90]
3	Upregulation of factors that inhibit apoptosis [97, 101-104].
4	Suppression of tumour cell proliferation thereby reducing sensitivity to chemotherapy [105].

At what point in time hypoxia becomes important to tumour development and propagation is unclear. The appearance of hypoxic markers 80μm from blood vessels within tumours suggests that a hypoxic microenvironment is likely to have developed by the time the diameter of a cluster of neoplastic cells reaches this size [106]. Several observations suggest hypoxia may even be present from the conception of the tumourigenic process. Hypoxia induced factors have been noted in early neoplastic change. Two studies have investigated the expression of the hypoxia inducible transcription factor, hypoxia inducible factor (HIF)-1α, at different stages of carcinogenesis. They found that little or no expression is seen prior to transformation but with each incremental increase in tumourigenesis there is an increase in expression of HIF-1α [107, 108]. Studies of papanicolaou smears and bronchial biopsies have also noted the HIF-1α regulated protein carbonic anhydrase (CA) IX is present in lesions demonstrating neoplastic change and has been proposed to be used as an adjunct to the present classification that relies on morphological changes only [109, 110].

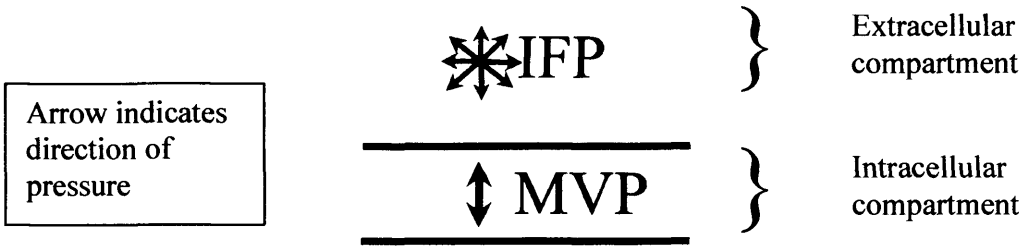
1.3. DEVELOPMENT OF A HYPOXIC TUMOUR MICROENVIRONMENT

The causes of tumour hypoxia have been partially elucidated (table 1.4.). Hypoxia may be a result of either acute or diffusion limited hypoxia. Acute hypoxia describes episodes of reduced tissue perfusion. Diffusion limited hypoxia occurs as a result of tumour cells being situated at a greater distance from blood vessels than the diffusion distance of the arteriolar pO_2. Both these phenomena are commonplace in the tumour milieu [111].

Reduced perfusion occurs as a result of an aberrant circulatory structure. The tumour microvasculature is characterised by irregular formations such as blind loops, coils and ribbons, which may cause shunting of the circulation. Microvessels are excessively long and areas of vessel compression are frequent [111, 112]. Poisseille's law states that excessive length of vessels will require increased arteriolar pressure to perfuse. Accordingly blood flow within tumours has been seen to be intermittent when windows have been placed in xenograft tumours [111, 113, 114].

The relative immaturity of tumour vasculature may be partly responsible for these abnormal features. Tumour endothelial cells often lack supporting structures compared to physiologically normal endothelium [115, 116]. The lack of mature vessels may be due to the shortened survival of tumour vasculature. The invading front of tumours have a higher vessel density than the relatively avascular centre of tumours, which suggest tumour microvessels have a short life span [117, 118]. This phenomenon creates a disproportionate dependence on the angiogenic process. However some normal tissues also have an increased dependence on angiogenesis such as the ovarian corpus luteum but are able to compensate by means of a higher rate of endothelial cell proliferation than that observed in the tumour environment [116].

Tumour interstitial fluid pressure (IFP) is higher than in normal tissue due to increased permeability of the vasculature and the absence of a lymphatic system within the tumour. Raised IFP was initially thought to contribute to compression of tumour vessels thereby causing acute hypoxia but this appears not to be the case [119]. IFP is dependent on the mean vascular pressure (MVP) and hence cannot exceed MVP, which would need to be the case if the IFP were responsible for vascular collapse (figure 1.2.) [120].



If IFP depends on MVP then IFP ≤ MVP

Figure 1.2. Interstitial fluid pressure is dependent on mean vascular pressure

The inefficiency of the tumour microcirculation is often exacerbated by a reduced capacity to transport O_2 from the lungs to the tissues in cancer patients. Such instances are commonplace due to an absolute or functional anaemia. Functional anaemia is common in lung cancer due to cigarette smoking induced carboxyhaemoglobin levels.

Table 1.4. Recognised causes of tumour hypoxia

1	Increased diffusion distances
2	Irregular vascular architecture
3	Immaturity of vasculature architecture
4	Lack of auto-regulation of blood flow
5	Inefficient angiogenesis
6	Reduced vascular survival
7	Anaemia-functional or absolute
8	Increased interstitial fluid pressure; possible

1.4. HYPOXIA INDUCIBLE FACTORS

1.4.1. Hypoxia inducible factor-1α

HIF-1 has been identified as the "master" transcription factor that orchestrates the cellular response to hypoxia and is overexpressed in solid tumours. HIF-1 is a heterodimer that consists of 2 subunits, HIF-1α and HIF-1β [92]. HIF-1β is also known as the aryl hydrocarbon nuclear translocator (ARNT).

Both subunits are constitutively expressed in the cytoplasm under normal physiological conditions. The levels of HIF-1α are suppressed by ubiquitination, which is a multi-step process that labels proteins for uptake and subsequent digestion by proteosomes. The process is dependent on the Von Hippel Landau protein (pVHL) that acts as a bridging protein between the HIF-1α molecule and the ubiquitin ligase complex. Under hypoxic conditions VHL fails to bind to HIF-1α, thereby allowing HIF-1α to accumulate in the cytoplasm [121, 122]. The transcriptional activity of HIF-1 is also regulated by O_2 independent processes, which may influence the rate of synthesis and transcriptional activity.

Stabilised HIF-1α binds to HIF-1β and undergoes a conformation change, that increases HIF-1α's DNA affinity [123]. The HIF-1 dimer subsequently translocates to the nucleus where it binds to a specialised genomic sequence, known as the hypoxia response element (HRE) in the DNA promoter region of target proteins. Comparison of ten HIF-1 binding sites identified within HREs from known target proteins revealed an invariant core sequence 5'-CGTG-3' [92].

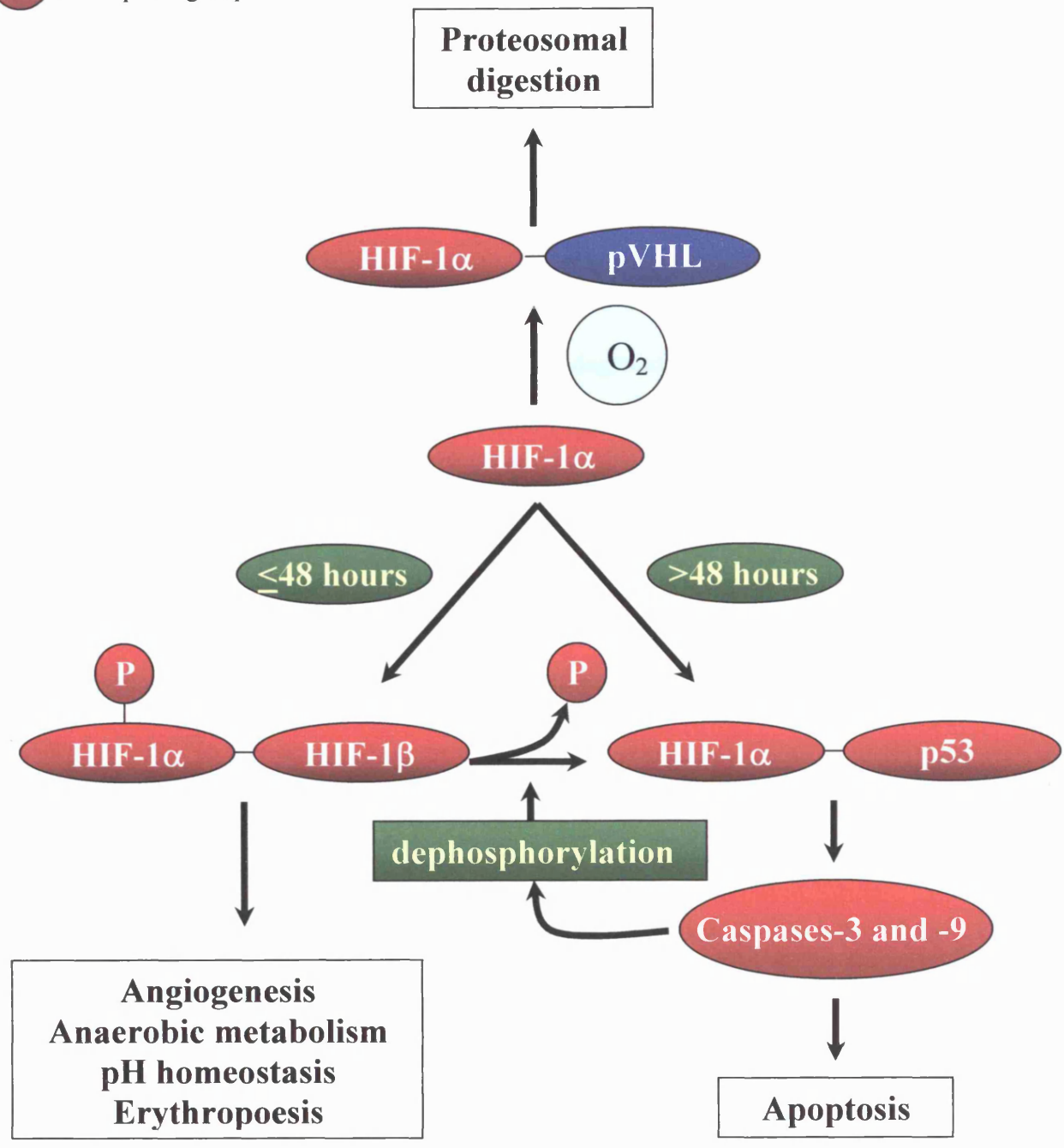
HIF-1 target proteins facilitate tumour cell survival in an otherwise hostile environment through angiogenesis, suppression of the immune response, modulation of the microenvironment pH and increasing anaerobic metabolism [92, 124]. Conversely HIF-1 also up-regulates and stabilises proteins that induce apoptosis and growth arrest. Gene deletion studies have predominantly reported that tumour growth and angiogenesis is suppressed in the absence of HIF-1 activity [125-128]. One study has reported the opposite for the effects on tumour growth but the effects on angiogenesis were the same [129].

The Janus faced nature of HIF-1 has been examined by a number of studies. Work in breast cell lines suggests that the level of HIF-1α induction determines whether this transcription factor acts as a positive or negative tumour growth factor. Cell lines that have either a high or low induction of

HIF-1α in response to hypoxia have a low metastatic potential in contrast to cell lines with moderate induction of HIF-1α that have prolonged clonogenic survival under hypoxic conditions [130]. Suzuki *et al.* studied breast cancer cell lines exposed to hypoxia for different time intervals and found that apoptosis increased after 48 hours of exposure. This time frame coincided with an alteration in the phosphorylation status of HIF-1α with an increase in the dephosphorylated form. Immunoprecipitation studies demonstrated that phosphorylated HIF-1α binds to HIF-1β to form the HIF-1 dimer. In contrast, dephosphorylated HIF-1α binds to and stablilises p53, activating the p53 dependent pro-apoptotic pathway. A feedback loop was found where end products of the p53 pro-apoptotic pathway inhibited phosphorylation of HIF-1α. Hence induction of HIF-1α by prolonged hypoxia in the presence of wildtype p53 may lead to increased apoptosis (figure 1.3)[131].

Figure 1.3. Factors determining the actions of HIF-1α

P : Phosphate group



As previously stated HIF-1α expression has been shown to increase incrementally through advancing stages of tumourigenesis [107, 108]. These observations suggest that HIF-1 plays a role in promoting malignant change but do not exclude the increased expression being the result of a bystander effect. Evidence for the former is seen in a number of conditions where increased HIF-1α expression has a direct causative role. In the inherited cancer syndrome, Von Hippel Lindau disease, which is characterised by an increased incidence of highly vascular tumours, pathological up-regulation of HIF-1α occurs in the absence of hypoxia. The primary lesion is a mutation in the pVHL oncosuppresser gene that prevents normoxic degradation of HIF-1α through ubiquitination [132]. Pathological increased expression of HIF-1α has also been suggested to have an aetiological role in Kaposi's sarcoma and cervical carcinoma. Kaposi's sarcoma associated virus/ human herpes virus 8 codes for a constitutively active G protein-coupled receptor that stimulates HIF-1α activity [133]. In cervical cancer, human papilloma virus codes for the E6 oncoprotein, which has been shown to stimulate increased expression of HIF-1α as a consequence of reduced levels of active wildtype p53 [126].

HIF-1α expression has been shown to be up-regulated in a variety of solid tumours, including NSCLC, squamous cell carcinoma of the head and neck (SCCHN), oligodendromas, breast and cervical cancer [134-143]. The majority of immunohistochemical studies have reported increased HIF-1α to be associated with a poor prognosis but this is not a universal finding. Indeed in early stage head and neck cancer treated with surgery, the presence of HIF-1α correlated with a good prognosis [142].

A more in depth description of the molecular structure and events regulating the transcriptional activity is included in the introduction to chapter 5.

1.4.2. Hypoxia inducible factor-2α

HIF-2α is a transcription factor that shares close structural homology with HIF-1α [144, 145]. Like HIF-1α, this transcription factor binds to HIF-1β, is able to stimulate transcription of genes containing a HRE and is regulated by pVHL dependent proteosomal degradation [144, 146, 147]. Also, as for HIF-1α, O_2 independent processes have a role in regulating transcriptional activity of HIF-2α. For instance, phosphorylation of threonine 822 is necessary for activation of the protein independently of the degree of hypoxia [148]. The cell types expressing HIF-2α and HIF-1α differ. HIF-2α is expressed by tumour-associated macrophages (TAM) often in the absence of tumour cell expression [149].

HIF-2α appears to play an important role in angiogenesis. In chick embryonic angiogenesis, deletion of the gene causes early death in utero accompanied by severe vascular defects [150]. In solid tumours angiogenesis has been correlated with HIF-2α positive TAM in breast cancer and in HIF-2α positivity (combined tumour cell and macrophage expression) in NSCLC [141, 151]. Macrophages are differentially attracted to areas of hypoxia where upregulation of HIF-2α may stimulate release of vascular endothelial growth factor (VEGF) thereby offering an explanation of the relationship between increased macrophage infiltrate and angiogenesis observed in breast cancer [152]. Studies in head and neck and bladder cancer have failed to replicate the relationship between HIF-2α and angiogenesis [142, 153]. The apparent contradictory nature of these results is not surprising considering the complexity of factors regulating angiogenesis. Furthermore, these studies do not exclude hypoxia from having an important role in stimulating angiogenesis in these tumour types as a number of hypoxia inducible angiogenic factors exist that are not regulated by HIFs (see later). HIF-2α also induces apoptosis in response to hypoglycaemia but not in response to hypoxia [154].

A similar picture to that of HIF-1α exists for the relationship between HIF-2α and prognosis. HIF-2α expression predicts a poor prognosis in NSCLC and a marginal association has been found in breast cancer but no association was found in head and neck cancer [141, 142, 151]. The contradictory nature of the associations with prognosis suggests a similar heterogeneity in the actions of HIF-2α to that of HIF-1α with regard to orchestrating tumour cell survival processes or apoptosis.

1.5. APOPTOSIS

1.5.1. Mechanisms of hypoxia induced apoptosis

Under normal physiological conditions, tissue hypoxia is rare due to local and systemic regulatory processes that increase blood flow and respiratory rate in response to increased oxygen demand [155]. Vital cellular processes e.g. synthetic reactions, muscular contraction, nerve impulse conduction and active transport require free energy of which adenosine triphosphate (ATP) is the body's main reservoir. Oxidative phosphorylation within mitochondria generates the majority of ATP and is dependent on O_2 [156]. Under prolonged hypoxic conditions the rate of ATP synthesis is decreased leading to reduced capacity for vital processes, which results in a drop in the intracellular pH and subsequent apoptosis [157].

Apoptosis is a process of programmed cell death that results in compartmentalised fragmentation of the cell thereby causing cellular suicide without eliciting an inflammatory reaction [158, 159]. Apoptosis is initiated either by a mitochondrial pathway or by activation of death receptors (figure 1.4.). Proteins in the mitrochondrial pathway are cytochrome c, apaf-1, BAX, BAD, Bcl-2 and the caspases-3 and 9. Under hypoxic conditions the mitochondrial pathway is activated by the release of cytochrome c, which combines with apaf-1 to up-regulate caspases-3 and 9 that trigger apoptosis. Comparison of cell lines genetically matched at the genetic loci of members of the mitochondria

pathway found the apaf-1 and the caspase genes are essential for apoptosis under hypoxic conditions. In contrast the tumour suppressor p53 was not found to be an essential component [160].

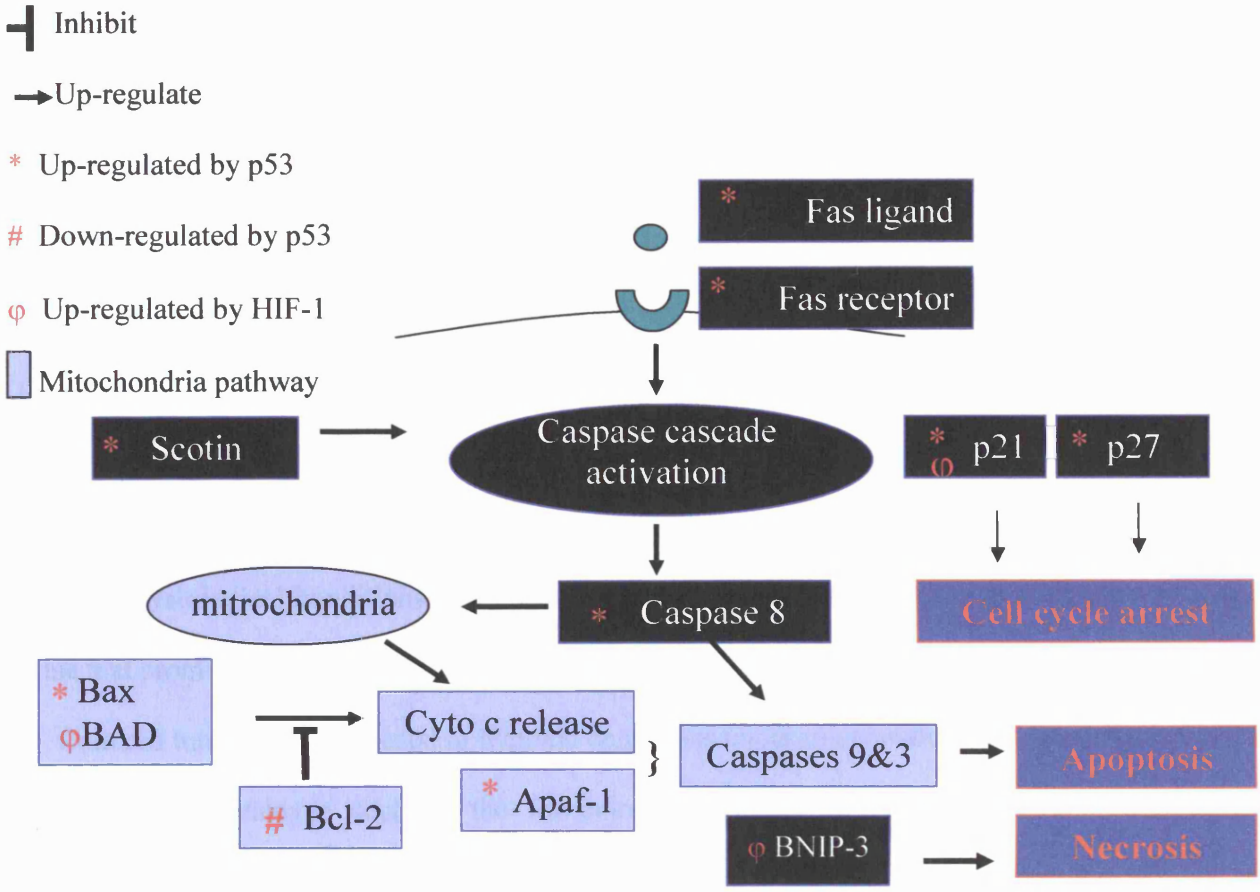


Figure 1.4. Apoptotic pathways

P53 regulates the magnitude of apoptosis by increasing transcription of Bax that increases cytochrome c release and cytoplasmic levels of apaf-1 [161]. P53 is usually continuously degraded but is stabilised under hypoxic conditions in part by the binding of HIF-1α as has been previously mentioned [131].

HIF-1α can also trigger cell death independently of p53. HIF-1α up-regulates two pro-apoptotic members of the Bcl-2 family of mitochondria proteins Bcl-2/adenovirus EIB 19kD-interacting protein 3 (BNIP3) and Nip 3 like protein X (NIX) [162]. BNIP-3 causes necrosis like cell death as opposed to apoptosis by opening the mitochondria permeability pores that induce profound

mitochondria dysfunction [163]. The pro-apoptotic protein BAD is also indirectly up-regulated by HIF-1α. Expression of BAD is suppressed by Insulin-like growth factor (IGR)-1 signalling through the IGF-1 receptor (IGF-1R). HIF-1α stimulates expression of IGF-1 binding protein-3, which blocks IGF-1R activation [164].

Hypoxia can also induce a dormant state by preventing continual replication. This is performed through induction of the cyclin dependent kinase inhibitors p21 and p27 [165, 166]. Separate gene deletion studies have consistently reported a role for HIF-1α in the regulation of p21 but differ with regard to the regulation of p27 [129, 166].

1.5.2. Chronic hypoxia results in DNA and chromosomal alterations that inhibit apoptosis

Due to induction of apoptosis and cell cycle arrest, prolonged hypoxia is an unusual event under normal physiological conditions. If cells do survive there is evidence that genetic mutations may arise that promote tumourigenesis.

Cultured tumour cells exposed to hypoxic conditions undergo anomalous DNA synthesis that can result in DNA damage such as the formation of fragile sites and subsequent chromosomal amplification [167-169]. Chromosomal amplification of oncogenes can increase their activity and promote tumourigenesis.

Hypoxia selects for cells with p53 mutations as similar cells, expressing wild-type p53 tend to undergo apoptosis [90]. P53 initiates apoptosis in response to DNA damage as well as to hypoxia and so protects against development of a malignant clone. A vicious circle may arise as hypoxia selected cells with p53 mutations may be more susceptible to hypoxia induced DNA damage thereby increasing the likelihood of malignant transformation.

A further example of how hypoxia may promote tumourigenesis is by the induction of the telomerase enzyme [170]. Under normal conditions, any cell of non-germ line lineage may only undergo a finite number of replication cycles. This is due to the fact that the chromosomes are

capped by telomeres that shorten with each cell cycle until a set length is reached when apoptosis is triggered. Telomerase lengthens the telomere enabling immortalisation of the cell, a fundamental characteristic of malignant transformation [171].

1.6. HYPOXIA AND MICROENVIRONMENTAL ACID/ BASE BALANCE

In 1930 it was noted that tumour cells had a remarkable capacity to convert carbohydrate to lactic acid [172]. Microelectrode measurements of tumours confirmed a relatively low pH. Subsequently, ^{31}P magnetic resonance spectroscopy enabled the intracellular pH (pHi) to be estimated and the pHi was shown to be neutral or slightly alkaline. Hence the normal pH gradient across the cell membrane in the tumour milieu is reversed, where the extracellular pH (pHe) is lower than the pHi [173]. Initially the acidic pHe of tumours was thought to be a consequence of increased usage of anaerobic metabolism resulting in increased lactic acid levels. However studies with solid tumour xenografts grown from cell lines with mutant glycolytic enzymes also reported a similar acidic pHe [174, 175]. Other causes of the acidic tumour pHe needed to be sought.

A further explanation is that the acidic pHe could be attributed to either an increased H^+ ion secretion by tumour cells and, or by adjacent host cells possibly as a result of growth factors or cytokines secreted by tumour cells. An example of the former has been identified, overexpression of CA isoenzymes lower the pHi *in vitro* [176]. The CA enzyme family catalyse the hydration of carbon dioxide to carbonic acid thereby acidifying the media and buffering the intracellular compartment [177].

$$CO_2 + H_2O \longleftrightarrow HCO_3^- + H^+$$

CA IX and XII have been identified as hypoxia inducible membrane bound isoenzymes. CA IX is regulated by HIF-1 and has been associated with a poor prognosis in solid tumours [124, 178-180].

A low pHe in the main is beneficial to tumour progression. It promotes tumour invasion in part by the induction of endopeptidases that break down the extracellular matrix facilitating tumour cell spread [181]. A low pHe also suppresses the host immune system by inhibiting the anti-tumour affects of lymphokine activated killer and peripheral blood mononuclear cells [182, 183]. However under hypoxic conditions tumour acidity further up-regulates p53 dependent apoptosis. Buffering these pH changes *in vitro* inhibits apoptosis by down-regulating p53 and enables survival in an otherwise hostile microenvironment [157]. Membrane bound CA may thereby promote tumour progression by acidifying the pHe and protecting the tumour cell from apoptosis by buffering the pHi.

The pHe may also influence the passive transport of anticancer drugs across the cell membrane depending the pK of the drug. For example those drugs with a lower pK will be transported across in their non-ionised form i.e. 5FU. Those with a higher pK will be ionised and therefore relatively hydrophilic and not cross the cell membrane i.e. adriamycin [184].

1.7. HYPOXIA AND TUMOUR CELL METABOLISM

A striking characteristic of malignant cells is their capacity to take up and metabolise glucose to lactic acid as a means of generating ATP [185]. For this purpose the tumour cell is reliant on increased expression of glucose transporters and up-regulation of the enzymes involved in glycolysis. HREs have been identified in the promoter regions of several enzymes involved in the glycolytic process including lactate dehydrogenase (LDH) A, enolase (ENO) 1, aldolase A, type II

hexokinase, glucose transporter GLUT-1, phosphoglycerate kinase 1, pyruvate kinase M and phosphofructokinase [186-189].

Like HIF-1α and CA IX, GLUT-1 increases in expression through the different stages of tumourigenesis [107]. Increased expression of anaerobic enzymes conveys a protective effect to tumour cells under hypoxic and hypoglycemic conditions and promotes tumour cell proliferation [190, 191]. It is likely that these survival mechanisms promote tumour spread as higher levels of expression of anaerobic enzymes have been reported in primary tumours that metastasize early and in metastatic lesions compared to their corresponding primary tumours [188, 192].

1.8. ANGIOGENESIS

Over the last 30 years angiogenesis has been increasingly recognised as an essential process for carcinogenesis, tumourigenesis and metastatic spread, to which a great debt must be paid to J Folkman and colleagues [193]. Angiogenesis is required for a tumour to grow more than 2mm in diameter. Prior to this in the "pre-vascular stage" they grow in a slow linear fashion [193]. A study of bronchial biopsies ranging from normal tissue through to invasive lesions found that microvessel density (MVD), a surrogate marker of angiogenesis only increased at the dysplastic stage [194]. Angiogenesis is equally important to the growth of metastases as it is to the development of the primary lesion. In the absence of angiogenesis, tumour cell proliferation within these lesions is matched by tumour cell apoptosis, thereby preventing tumour growth [195]. The development of new blood vessels facilitates tumourigenesis by the delivery of nutrients and disposal of metabolic waste and provides a portal for the dissemination of tumour cells thereby playing a central role in the metastatic spread of malignant disease. Angiogenesis has been correlated with a poor prognosis in many different tumour types [196]. In our laboratory this has been reported in NSCLC and mesothelioma by evaluating the MVD by using a Chalkley graticule to evaluate hot spots [196-198].

The angiogenic process is a balance between stimulatory and inhibitory factors. An imbalance in favour of the stimulatory factors will lead to tripping of the "angiogenic switch" inducing microvessel growth. A multitude of stimulatory and inhibitory factors have been identified. Hypoxia may both promote stimulatory and suppress inhibitory angiogenic factors.

1.8.1. Hypoxic upregulation of angiogenic growth factors

1.8.1.1. Vascular endothelial growth factor

Vascular permeability factor also termed vascular endothelial growth factor VEGF is the founder member of a family of closely related cytokines that exert critical functions during vasculogenesis and in both pathological and physiological angiogenesis and lymphangiogenesis. Initially discovered in the late 1970's, VEGF was found to be a potent vascular permeability factor. Since that time VEGF has been recognised to be an endothelial and tumour cell mitogen, migratory and anti-apoptosis factor and immune suppressor [199-202]. Multiple forms of VEGF exist that are encoded by a single gene and arise from alternative splicing of the RNA transcript [203-205].

VEGF stimulates angiogenesis indirectly by increasing vascular permeability. Increased vascular permeability results in the leakage of plasma proteins that including clotting factors such as fibrinogen [206-208]. These proteins rapidly activate the clotting cascade by means of the tissue factor pathway and this results in the deposition of extracellular fibrin [209, 210]. Fibrin transforms the properties of tissue stroma from anti- to strongly pro-angiogenic [209].

VEGF acts directly as an endothelial cell mitogen by binding to VEGF receptor (R)-2 (KDR/Flk-1) located on endothelial cells, which is a tyrosine kinase (TK) receptor [211]. Impedance of VEGFR-2 signalling arrests angiogenesis with subsequent tumour cell apoptosis and tumour

regression in treated mice [212-215]. VEGFR-1 (Flt-1) is also a TK receptor but its activity is involved in endothelial cell differentiation as opposed to proliferation [216-218].

VEGF also binds to two non-TK receptors, neuropilin (NRP)-1 and NRP-2, which have previously been described as having a role in guidance of growing neurons. They are expressed by tumour cells and NR-1 and –2 expression has been positively correlated with angiogenesis in NSCLC [219]. Their role appears to facilitate VEGR-VEGFR binding by forming complexes with certain VEGF isoforms [220, 221].

VEGF suppresses the immune response by blocking maturation of dendritic cell progenitors. Dendritic cells act as antigen-presenting cells involved in cell mediated immunity (CMI) and are thereby a linchpin in the body's anti-tumour armoury [91, 222]. VEGF may also stimulate differentiation of these progenitor cells into endothelial cells thereby assisting angiogenesis [223-226].

Hypoxia induces VEGF and VEGFR-1 but not VEGFR-2 [227, 228]. The transcription factors HIF-1, HIF-2, NF-kappa B and activator protein (AP)-1 all regulate VEGF under different conditions. Hypoxic induction of VEGF at a transcriptional level is dependent on HIF-1 or HIF-2 activation of the HRE [144, 229]. Deletion of the HRE sequence abrogates *in vivo* embryonic stem cell VEGF expression. Deletion of the HIF-1α gene halves VEGF expression. Hence a greater reduction in VEGF synthesis results from deletion of the HRE than from abrogation of HIF-1 activity [230]. This is possibly due to ongoing HIF-2 stimulated transcription of VEGF in the latter scenario. NF Kappa B is also induced by hypoxia, but a study in glial cells found inhibition of NF Kappa B activity had no effect on the hypoxic regulation of VEGF [231]. AP-1 binds to a DNA region upstream of the HRE, which although not essential for transcriptional activation potentiates VEGF induction by HIF-1 [232, 233]. These studies suggest that although a number of transcription factors are involved in VEGF regulation, HIF-1 and 2 are the dominant regulatory factors under

hypoxic conditions. Also a close correlation has been reported between the expressions of both HIF-1α and HIF-2α expression and VEGF in NSCLC [141]

The crucial role that VEGF plays in tumourigenesis is highlighted by a myriad of studies, which report that increased VEGF expression is associated with increased levels of angiogenesis, resistance to chemotherapy and hormone therapy and shorter disease free and overall survival [234-240]. In agreement with these studies *in vivo*, studies have found that inhibition of VEGF activity by targeted monoclonal antibodies (soluble VEGF receptors that sequester VEGF and VEGFR inhibitors), result in suppression of angiogenesis, tumour growth and metastatic spread [241-247]. As discussed earlier these experimental drugs have been effective in the treatment of solid tumours in the setting of clinical trials [77].

1.8.1.2. Other Angiogenic growth factors that are induced by hypoxia

A number of other angiogenic growth factors have been identified that are induced by hypoxia. These factors include tissue factor, platelet derived endothelial cell growth factor (PDECGF), interleukin (IL)-8, basic fibroblastic growth factor and urokinase plasmingogen activator (uPA) and its inhibitor plasminogen activator inhibitor (PAI)-1 [248-253].

HIF-1α is involved in the regulation of some, but not all of these angiogenic growth factors such as tissue factor and IL-8 [254-256]. Hypoxia stimulates the release of basic fibroblastic growth factor (bFGF) from different cell types within the tumour milieu and sensitises endothelial cells to bFGF activity [257-260]. This sensitisation is due to HIF-1α dependent synthesis of heparan sulfate co-receptors for bFGF [261]. No definite regulatory role has been identified for HIF-1 in the regulation of PDECGF but a strong association between both HIF-1α and HIF-2α with PDECGF in NSCLC has been reported [141]. uPA and PAI-1 act synergistically to promote angiogenesis [262-264]. A HRE has been identified in the PAI-1 gene promoter region and inhibition of HIF-1α

degradation results in induction of PAI-1 [265-268]. Interestingly uPA levels were found to decrease in the presence of mutated VHL even though they have been found to correlate with hypoxia in tumours [267, 269].

1.8.2. Does hypoxia suppresse anti-angiogenic factors?

As a swing in the balance between pro- and anti-angiogenic factors is required for angiogenesis to proceed, if hypoxia were pro-angiogenic it would be expected to suppress anti-angiogenic factors. A number of studies have investigated the effects of hypoxia on thrombospondin (TSP)-1, which is a secreted adhesive glycoprotein that has been shown to have anti-angiogenic properties. The number and results of these studies are relatively sparse and inconsistent when compared to studies investigating pro-angiogenic factors. Hypoxia has been demonstrated to reduce, have no effect and to increase levels of TSP-1 in glioblastoma, bladder and endothelial cell lines respectively [270-272]. A study by Laderoute *et al.* looked at a series of transformed and untransformed cell lines and reported that hypoxic suppression of TSP-1 in normal cells was cell specific. Transformation itself considerably suppressed TSP-1 but where TSP-1 expression was still detectable, hypoxia was able to confer further suppression [273]. They concluded that hypoxia, in certain circumstances, augments the effects of oncogenic signalling with regard to TSP-1 regulation. TSP-2 and angiostatin are other naturally occurring anti-angiogenic factors but no evidence exists as to whether hypoxia plays a role in their regulation.

1.9. PROCESSES THAT ENHANCE THE HYPOXIC RESPONSE

1.9.1. Cross talk between hypoxia and non-hypoxia related signalling

There is increasing evidence that the cellular response to hypoxia is modified by oncogenes, cytokines and growth factors. These factors signal via different intracellular pathways. The phosphatidylinositol 3-kinase (PI3K), mitogen associated protein kinase (MAPK), and Smad pathways have the capacity to influence the expression of HIF-1α by oxygen independent processes. These pathways are overactive in a wide range of tumour types. The end products are able to influence a repertoire of responses ranging from cell growth and proliferation to survival and motility that drive tumourigenesis.

1.9.2. Epidermal growth factor receptor and PI3K pathway

PI3K activates protein kinase B (also known as Akt). PKB was identified by 3 groups simultaneously in the early 90's and was named PKB due to its homology with protein kinases A and C. In humans three PKB genes have been identified, PKBα, PKβ and PKγ. All PKB isoforms are composed of an N-terminal pleckstrum homology (PH) domain, a central kinase domain and a C-terminal hydrophobic regulatory domain (figure 1.5).

The PI3K pathway is initiated by activation of growth factor receptors that possess tyrosine kinase activity such as EGFR, G-protein-coupled receptors or by direct interaction with the Ras proto-oncogene. Phosphorylation of the intracellular tyrosine residues results in translocation of the PI3K from the cytoplasm to the membrane. The PI3K enzyme consists of an 85KD domain and a 110 KD domain. The 85KD domain is phosphorylated by the receptor tyrosine kinase and the 110 KD domain catalyses the transfer of phosphate from ATP to the D-3 position of the inositol ring of the membrane bound phosphoinositides, thereby generating 3'4'-phosphatidyl-biphosphate and 3'4'5'- phosphatidyl–triphosphotides. These phosphoinositides bind to the PKB PH domain thereby

recruiting PKB to the membrane. The kinase that phosphorylates PKBα is called 3-phosphoinositide-dependent kinase-1 as it requires the presence of phosphoinositides to induce a conformational change in PKBα. The conformational change exposes the catalytic loop allowing phosphorylation of threonine 308, which partially activates PKBα. For full activation, phosphorylation of serine 473 in the regulatory domain is required. The processes involved have not been fully elucidated but may involve a second kinase or a negative feedback loop from activated PKBα (figure 1.6.).

The PI3K pathway is negatively regulated by the tumour suppressor lipid phosphatases, **p**hosphatase and tensin homolog deleted on chromosome **ten** (PTEN) and **SH**2-domain-containing **i**nositol **p**hosphatase (SHIP). These phosphatases dephosphorylate PIs at the 3' and 5' positions respectively. Mutation of PTEN and SHIP genes has been reported in solid tumours and results in resistance to apoptosis. Active PKB in the cytoplasm is dephosphorylated by protein phosphatase 2A (PP2A). The information presented here has been gleaned from reviews by K. M. Nicholson and N. G. Anderson, M. M. Hill and B. A. Hemmings and by S.R. Datta, A. Brunet *et. al.* (figure 1.6.) [274-276].

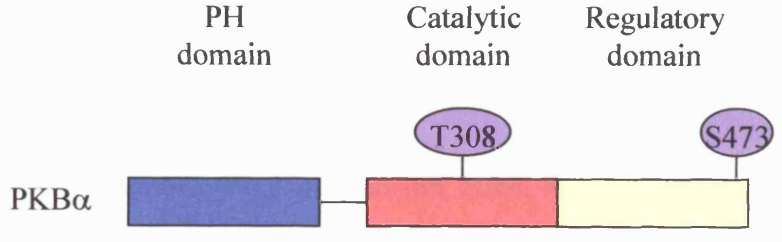


Figure 1.5. PKBα structure

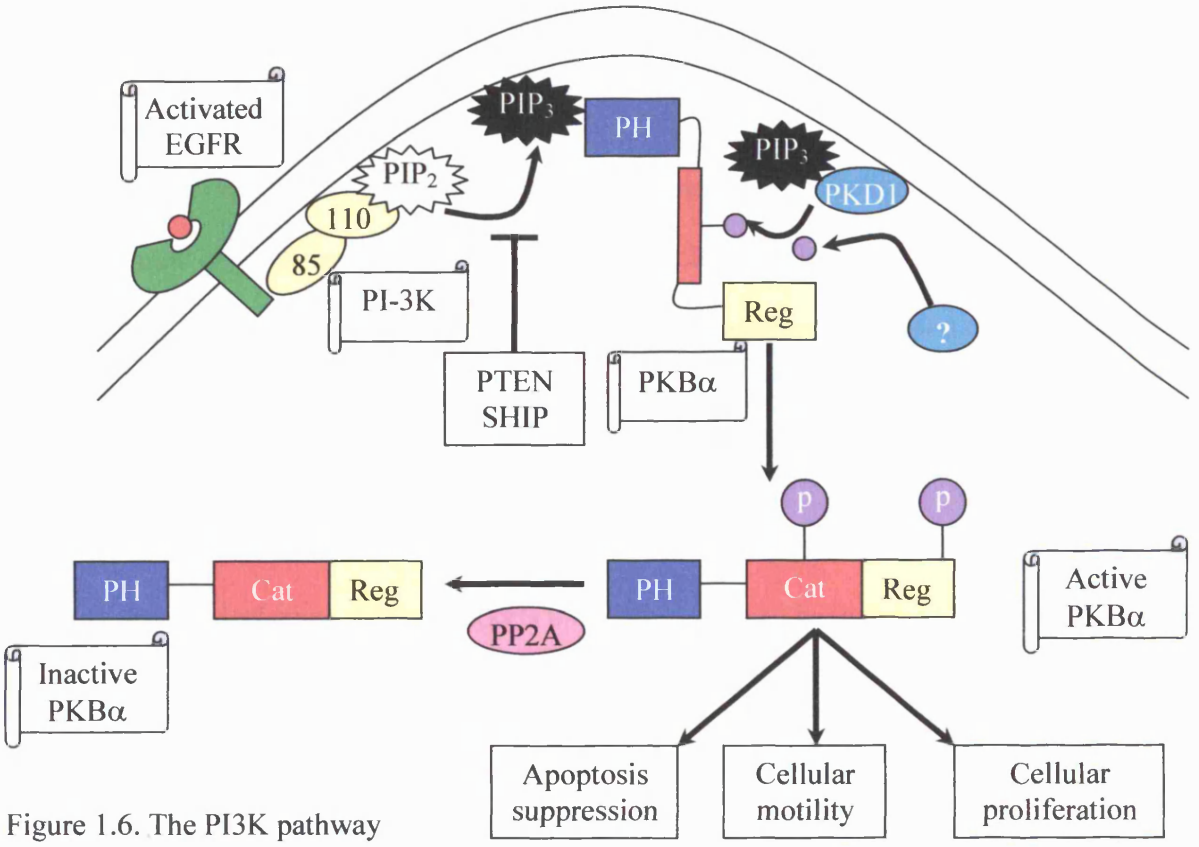


Figure 1.6. The PI3K pathway

The EGFR is a member of the c-erb family of tyrosine kinase receptors. This receptor is commonly overexpressed in NSCLC tumours and activation results in increased angiogenesis, tumour cell proliferation, invasion, resistance to chemotherapy and reduced apoptosis [64] (reviewed by Mendelsohn [277]). A number of studies suggest that activation of this receptor enhances the cellular response to hypoxia. EGF stimulation of prostate cell lines induces HIF-1α via the PI3K pathway independently of and additively to hypoxia [278]. Similarly in glioblastoma cells, activation of EGFR induced VEGF expression by the PI3K pathway [279]. A further study in lung adenocarcinoma cells found that constitutionally active EGFR signalling by the PI3K pathway increased the hypoxic levels of VEGF but did not effect normoxic VEGF levels [280]. These studies suggest that EGFR related activation of the PI3K pathway enhances the hypoxic induction of angiogenesis.

A number of other growth factors, cytokines, oncogenes, cellular conditions and mutations have been reported to stimulate the PI3K pathway and increase HIF-1α and, or expression of its target

proteins. These studies have mostly been performed in prostate cancer cell lines but have also been performed in breast cancer, hepatoma and HeLa cell lines [281-291].

The picture is not totally consistent, as 2 recent studies have reported that the effects of activation of the PI3K pathway on HIF-1α expression and transactivatory activity are cell line specific and of a smaller magnitude to that which is induced by hypoxia [292, 293]. However immunohistochemical studies in human tumours have found that loss of PTEN is associated with increased angiogenesis and pathological stage, which supports the aforementioned hypothesis [294, 295].

1.9.3. Mitogen activated protein kinase pathways

Mitogen activated protein kinase (MAPK)s are a family of protein kinases that form a number of related intracellular signaling pathways. These pathways are each composed of a phospho-relay unit composed of a triumvirate of protein kinases that are regulated by phosphorylation. MAPKs are the final leg of the phosphor-relay, the relay being initiated by activation of a MAPK kinase kinase (MKKK), which phosphorylates and activates a MAPK kinase (MKK) that likewise activates the MAPK. MAPK phosphatases reverse the phosphorylation and return the MAPK to its resting state. Different MKKKs and MKKs are highly specific in phosphorylating specific substrates. MKKKs have specific motifs in their sequences, which allow selective activation of certain MKKKs by set stimuli [296]. The function and regulation of these pathways have been conserved during evolution from unicellular organisms to complex organisms such as humans. Substrates include protein kinases, transcription factors and cytoskeletal proteins.

In multicellular organisms there are 3 well characterised MAPK sub-families. These include the extra-cellular signal related kinases (ERK)-1 and –2 (also known as p42 and p44 MAPK, respectively); the c-Jun NH_2-terminal kinases (JNK)-1, -2 and –3; and the four p38 enzymes, p38α, p38β, p38γ and p38δ [296, 297].

ERK1 and ERK2 are activated by a variety of stimuli including growth factors, cytokines, viral infections, G-proteins, carcinogens and transforming agents. Activation of this pathway results in tumour cell proliferation, angiogenesis and resistance to apoptosis [255, 296-299].

The JNKs were isolated and characterised as the stress-activated protein kinases on the basis of their activation in response to inhibition of protein synthesis. An important target of the JNK pathway is c-Jun, a component of activated protein (AP)-1. AP-1 contributes to the control of many cytokine genes and is activated in response to cellular stresses, radiation and growth factors. Regulation of the JNK pathway is extremely complex and there are at least 13 MKKKs that regulate JNKs. Inhibition of the JNK pathway renders cells resistant to apoptosis and as such it represents a target for enhancing the efficacy of chemotherapy [296, 297].

The p38 MAPK pathway is regulated by inflammatory cytokines in immune cells and has an important role in modulating the immune response. This pathway is expressed in most cell types and can be activated by hormones, activated G-protein coupled receptors, osmotic shock and heat shock proteins. The summary of the MAPK pathways were taken from two reviews by G. L. Johnson and R. Lapadat and by M. H. Cobb and E. M. Schaffer [296, 297].

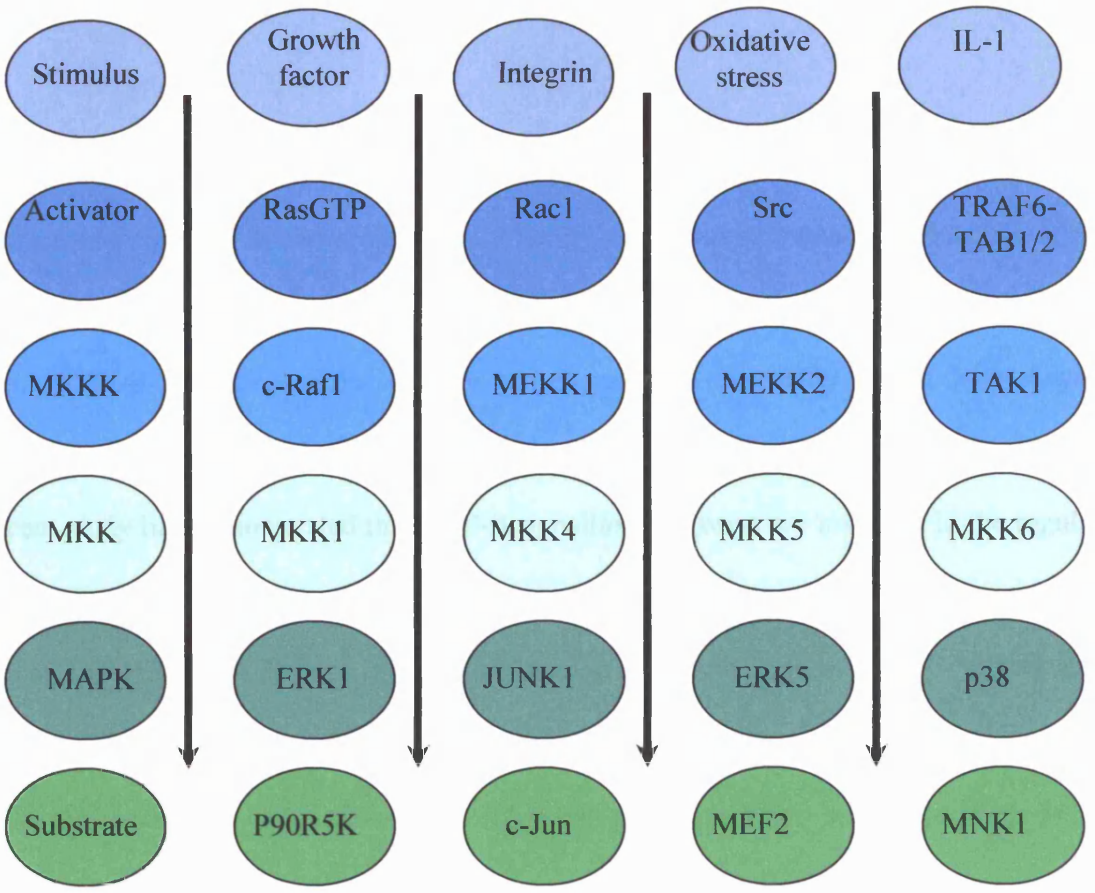


Figure 1.7. The MAPK pathways [297]

Stabilisation of HIF-1α is not sufficient for transcriptional activity [300]. A further phosphorylation step is required, which is regulated by MAPK pathways. Most studies have reported the ERK 1 and 2 pathway is the most important MAPK pathway involved [300-303]. Hypoxia activates the ERK 1 and 2 pathway through increased intracellular calcium [303]. Activation of the p38 MAPK pathway by viral oncogenes and carcinogen induced free radicals has also been reported to phosphorylate HIF-1α and enhance transcriptional activity [133, 304].

1.9.4. Transforming growth factor-β and Smad family

TGF-β is the prototype of a large superfamily of secreted signalling polypeptides with a diverse range of functions. With relation to tumourigenesis the function changes in a temporal fashion. Initially TGF-β suppresses tumour cell proliferation. Later, when tumour cells become resistant to these growth inhibitory signals, TGF-β supports tumourigenesis, angiogenesis and metastatic spread. Secretion of TGF-β by tumour cells aids avoidance of the host immune system by promoting pro-

apoptotic signals in circulating B and T lymphocytes and macrophages. TGF-β signals by activating Smad transcription factors. Smad proteins are a family of tumour suppressors. Mutation of Smad proteins, particularly Smad 2 and 4 may enable tumour cells to escape Smad related pro-apoptotic signalling. TGF-β may also signal via non-Smad pathways especially in the later stages of tumourigenesis (reviewed in [305, 306]).

A recent study has demonstrated that TGF-β signalling pathways are involved in the regulation of VEGF. A DNA binding sequence for Smads was found within the promoter sequence of VEGF upstream of the HRE. TGF-β or hypoxia alone was found to induce VEGF. However the combination of hypoxia and TGF-β had an additive effect with regard to the induction of VEGF suggesting the existence of a synergistic relationship. Consistent with this proposal, co-immunoprecipitation experiments by the same group show that HIF-1 physically interacts with Smads when binding to the VEGF promoter region [307].

Endoglin is a cell membrane glycoprotein overexpressed on highly proliferating endothelial cells of angiogenic vessels. Endoglin binds TGF-β and modulates the endothelial response to TGF-β [308]. Increased endothelial cell endoglin expression is strongly associated with a poor prognosis in NSCLC, suggesting that endoglin has a fundamental role in tumour related angiogenesis [309]. The same group that investigated TGF-β and hypoxia dependent regulation of VEGF recently found that TGF-β and hypoxia also additively co-ordinate expression of endoglin [310].

In addition to modulation of angiogenesis, TGF-β and hypoxia may have a synergistic effect on a tumour's metastatic potential. In human umbilical vein endothelial cells, trophoblasts and breast cancer cells, *in vitro* hypoxia increases cell invasion by increasing expression of uPA receptor (uPAR) by a haeme sensitive pathway [311-313]. A study using breast cancer cells has shown this process to be dependent on the suppression of tumour cell nitric oxide (NO) synthesis [313].

However, in macrophages and endothelial cells hypoxia induces NO synthesis thereby having no overall effect on the total NO levels. In the presence of TGF-β this induction of NO in macrophages and endothelial cells is inhibited, thereby suppressing the overall NO concentration and enhancing hypoxia induced tumour invasiveness [313, 314].

1.9.5. Cyclo-oxygenase-2

Cyclo-oxygenase (COX)-2 is one of a pair of enzymes that catalyse the rate-limiting step in arachidonic acid metabolism and prostaglandin synthesis. COX-1 is constitutively expressed and has a role in normal physiological processes such as cytoprotection of the gastric mucosa and maintenance of normal renal function in a compromised kidney [315]. COX-2 is up-regulated under stressful physiological conditions, such as in areas of chronic inflammation and hypoxia, and is widely expressed in pre-cancerous lesions and established tumours [315]. Prostaglandin (PG) E_2 is an important product of COX-2 activity and mediates many COX-2 dependent processes including suppression of apoptosis and the immune response and stimulation of angiogenesis [315, 316].

Both *in vitro* and *in vivo* studies have clearly demonstrated that non-steroidal anti-inflammatory drugs (NSAIDs) and selective COX-2 inhibitors have anti-proliferative activity against solid tumours and suppress angiogenesis provided the malignancy overexpresses COX-2 [317-320].

Hypoxia induces COX-2 in different cell types *in vitro* [321-323]. The level and duration of COX-2 expression varies. In tumour cell lines that have a high and persistent COX-2 response, an enhanced level of VEGF induction has been reported. This effect is HIF-1α dependent [321]. COX-2 activity increases hypoxia dependent stabilisation of HIF-1α [324]. The mechanism of action may be due to suppression of the pVHL protein. Such an effect has been reported in gastroendothelial cells [325]. These findings suggest that tumours with high COX-2 expression are likely to have an enhanced angiogenic response under hypoxic conditions.

1.10. MEASURING TUMOUR HYPOXIA

In order to generate therapeutic strategies and target suitable tumours, methods of analysing tumour hypoxia need to be developed. Several invasive and non-invasive techniques are available that are able to indirectly or directly measure tumour hypoxia. The gold standard is the polarographic probe, which directly measures the local pO_2 within a tumour [326]. Non-invasive techniques include nuclear magnetic resonance (NMR) spectroscopy, single-photon emission computed tomography (SPECT) and positron emission tomography (PET). NMR involves the administration of perfluorocarbons. Some perfluorocarbon's spin lattice times are sensitive to the pO_2 [327, 328]. This paramagnetic effect can be detected using NMR and hence can be used to non-invasively measure pO_2 *in vivo* [327]. SPECT and PET involve the administration of radiolabelled azomycin containing products that undergo bioreduction and sequestration in hypoxic cells [329, 330]. These non-invasive techniques may be able to provide a quantitative measure of tumour hypoxia.

To enable an estimate of hypoxia to be made in tissue sections, immunohistochemical techniques have used both exogenous and endogenous markers of tissue hypoxia. Several azomycin containing markers have been used as exogenous markers [331, 332]. These products are administered to the patient 24 hours prior to tumour resection and are sequestered in hypoxic cells. The identification of endogenous markers enables retrospective analysis of archival stored specimens. CA IX, glucose transporter (GLUT)-1 and HIF-1α have been proposed as endogenous markers of hypoxia on the evidence that they are induced by hypoxia [124, 140, 188]. Expression of these factors is observed in areas that are expected to be hypoxic such as around necrotic tissue. Comparative studies of CA IX and GLUT-1 with pimonidazole reveal a high degree of overlap of the staining patterns. Correlations have also been found between the expression of these markers and hypoxia measured

by a polarographic needle. The strongest correlation was found with CA IX ($r=0.5$, $p<0.001$) [124, 188]. As it is impossible to take a biopsy from the exact place where a measurement was obtained with a polarographic needle, a degree of variability would be expected when comparing these two methods.

1.11. HYPOXIA AS A THERAPEUTIC TARGET

Three separate approaches have made use of hypoxia as a therapeutic target. Firstly, inhibiting the processes that suppress tumour cell apoptosis may reduce tumour cell survival in a hypoxic microenvironment. Examples of these are the EGFR targeted therapies discussed in section 1.1.4.3. and monoclonal antibodies against the HER 2 protein (Trastuzumab) that also inhibit stimulation of either or both PI3K and MAPK pathways [333]. As has been discussed previously, COX-2 is induced by hypoxia and suppresses tumour cell apoptosis. Trials are under way investigating the use of COX-2 inhibitors in combination with adjuvant and palliative chemotherapy for NSCLC and in chemo-prevention in high risk populations [334]. Phase II studies have reported reductions in circulating VEGF levels, low toxicity rates and promising progression free and overall survival in response to COX-2 inhibitors combined with Docetaxel in pre-treated NSCLC patients [335, 336]. Sulfonamides inhibit CA isoenzymes and possibly induce apoptosis by interfering with the intracellular pH homeostasis [337]. CA IX has a different sensitivity to sulfonamides than other CA isoenzymes raising the possibility that CA IX selective inhibitors can be designed that would have selective cytotoxicity against tumour cells [338].

A second approach is to design cytotoxic agents that are activated in a hypoxic microenvironment. An early example is the bio-reductive agent, mitomycin c that has been used in the treatment of NSCLC for the past 2 decades [339, 340]. Attempts have been made to enhance the cytotoxicity of mitomycin c by acutely manipulating the oxygen dissociation curve, so increasing

tissue hypoxia but results have been disappointing [341]. A more recent example is tirapazamine. A phase III study in patients with advanced NSCLC, CATAPULT 1, reported the addition of tirapazamine to cisplatin improved response and survival although adding it to existing chemotherapy combinations has not improved outcome [255, 342, 343]. The targeting of genes that are induced by hypoxia with gene therapy is an attractive approach. Pre-clinical studies have successfully transfected hypoxia regulated expression vectors, which encode genes that activate prodrugs and confer hypoxia dependent sensitivity [344]. Studies are underway investigating whether CA IX can be used as a target for immunotherapy but no success has been reported to date [345].

The third approach is to reduce tumour hypoxia during treatment. This approach has been used mostly as an adjunct to radiotherapy. To date the use of perflurocarbons, blood transfusions, O_2 (normobaric and hyperbaric) and, or carbogen ($95\%O_2/5\%CO_2$) breathing during treatment have been disappointing [346]. An indirect method using nicotinamide combined with carbogen inhalation may be more promising and is being investigated as an adjunct to radiotherapy in the ARCON study [347]. The method of action of nicotinamide was initially thought to be vasodilatation. More recently *in vivo* studies have suggested that nicotinamide has an adrenergic effect, so stimulating the breakdown of glycogen stores and increasing the glucose availability. This would limit the need for aerobic metabolism and so reduce O_2 consumption within the tumour [346].

1.12. CONCLUSIONS AND AIMS OF THE STUDY

The combination of hypoxia and increased activity of the intracellular pathways discussed enables tumours to promote angiogenesis, suppress local and systemic cell mediated immune responses and prevent apoptosis. Darwin proposed that a hostile environment selects organisms that develop survival mechanisms thereby promote survival of the fittest. This paradigm can be applied to the tumour microenvironment that selects adaptive cells, which are able to survive where others undergo apoptosis or are killed by immune defenses. A greater understanding of tumour hypoxia and development of reliable markers of tumour hypoxia may improve tailoring of existing therapies and the development of future treatment strategies.

The aims of this study are to investigate the relationships between HIF-1α, HIF-2α, CA IX and tumour necrosis with a number of growth factors, endopeptidases and apoptotic factors in NSCLC. The expression of these factors will then be compared to patient outcome to examine the prognostic implications. The use of CA IX as a marker of tumour hypoxia will be further explored. Finally the regulatory factors and pathways governing CA IX expression will be studied.

Chapter 2

2.1. MATERIALS

2.1.1. Buffers

Buffers for tissue culture and immunohistochemistry (IHC) were prepared using 18Ω ultrapure water and distilled water, respectively. Where necessary, pH of solutions was altered using 12N HCl or 5M NaOH as appropriate.

2.1.1.1. For IHC:

i. **Tris buffered saline (TBS)** *Final concentration for working solution*

20x stock solution

In 500ml of distilled water the following were dissolved:

Tris 121.14g *100mM*

Sodium Chloride 160g *300mM*

Adjusted pH to 7.65 and made up to 1L with distilled water.

Working solution

Stock solution was diluted by factor of 20 with distilled water and made up to 1L of working solution then 10g BSA was added (omited if goat antibodies were used).

ii. **Ethlyenediaminetetraacetic acid (EDTA)** *Final concentration for working solution*

In 500ml of distilled water the following were dissolved:

EDTA 93.04g (30 minutes on a stirrer) *0.5M*

Adjusted pH to 8.0

iii. **20xCitric Acid Buffer** *Final concentration for working solution*

In 500ml of distilled water the following were dissolved:

Citric acid monohydrate 42g *0.01M*

Adjusted pH to 6.0 and made the volume up to 1L with distilled water.

iv. **Dako antigen retrieval solution** purchased from Dako, Ely, Cambridgeshire, UK

Diluted by 1:10 with distilled water and pH to 6.1 using concentrated HCL.

2.1.1.2. For Western Blotting

i. **Tris buffered saline (TBS) high salt** *Final concentration for working solution*

In 500ml of distilled water the following were dissolved:

Tris 60.5g *50mM*

Sodium Chloride 80g *150mM*

Adjusted pH to 7.65, made up to 1L with distilled water and stored at room temperature.

ii. **TBS low salt** *Final concentration for working solution*

In 500ml of distilled water the following were dissolved:

Tris 24.2g *20mM*

Sodium Chloride 80g *150mM*

Adjusted pH to 7.65, made up to 1L with distilled water and stored at room temperature.

iii. **10x Tris/glycine (T/G) buffer** *Final concentration for working solution*

In 500ml of distilled water the following were dissolved:

30.3g Tris *250mM*

144g glycine *2M*

Made up to 1L with distilled water and stored at room temperature.

iv. **Sodium dodecylsulphate (SDS) Running Buffer** *Final concentration for working solution*

In 500ml of distilled water the following were dissolved:

100ml 10x Tris/glycine buffer	*5mM/ 200mM*
10ml 10% w/v sodium dodecylsulphate	*0.1% w/v*

Made up to 1L with distilled water and stored at room temperature.

v. **10x Western blot lysis buffer** *Final concentration for working solution*

2ml 1M Tris (pH7.5)	*200mM*
3ml 5M NaCl	*1.5M*
200µl 0.5M EDTA	*10mM*
200µl 0.5M EGTA	*10mM*
1ml Triton X-100	*10% v/v*
250µl 1M sodium pyrophosphate	*25mM*
200µl 0.5M β-Glycerophosphate	*10mM*
1ml 100mM sodium orthovanadate	*10mM*
20µl 5mg/ml leupeptin	*10µg/ml*

This was made up to 10ml with distilled water for stock solution and stored at 4°C. For working solution the stock solution was diluted by factor of 10 with distilled water, to which 1mM phenylmethylsulfonylfluride was added immediately prior to use.

vi. **4x Western blot sample buffer** *Final concentration of working solution*

1.52g Tris	*12.5mM*
30ml glycerol	*60%*
4g SDS	*8%*
100mg bromophenol blue	*0.02% w/v*

Stock solution was stored at room temperature. 5% β-mercaptoethanol was added immediately prior to use in a 1:4 *v/v* ratio.

vii. **Transfer buffer** *Final concentration of working solution*

200ml methanol	*20%*
100ml 10x Tris/glycine buffer	*48mM Tris/ 38mM glycine*

Made up to 1L with distilled water.

viii. **Upper Gel Buffer** *Final concentration of working solution*

6.06g Tris	*0.5M*
4ml of 10% SDS stock	*0.4%*

pH to 6.8 with 1M HCL, made up to 100ml with distilled water, filtered through 0.45μm filter and stored at room temperature.

ix. **Lower Gel Buffer** *Final concentration of working solution*

45.41g Tris	*1.5M*
10ml of 10% SDS stock	*0.4*

Adjusted pH to 8.8, made up to 250ml with distilled water, filtered through 0.45μm filter and stored at room temperature.

x. **Electrode buffer** *Final concentration of working solution*

10ml of 10% SDS stock *0.1%*

100ml T/G

Made up to 1L with distilled water.

xi. **Stripping buffer** *Final concentration of working solution*

100ml of 10%SDS stock *1%*

24.78g Tris *0.2M*

Made up to 1L with distilled water, adjusted pH to 6.8and stored at room temperature. Prior to use add 40µl β-mercaptoethanol to 50ml of buffer.

2.1.2. Counterstain for IHC

Haematoxylin

In 2L of warm distilled water dissolve:

2g of haematoxylin

100g of potassium alum

100g of chloral hydrate

2g of citric acid monohydrate

400mg of sodium iodate

2.1.3. Primary antibodies

i. Mouse monoclonal anti-HIF-1α (H1α67, clone NB 100-123) IgG purchased from Novus, USA.

ii. Mouse monoclonal anti-HIF-1α ESEE 122 IgG1 and mouse monoclonal Anti-HIF-2α EP190b mouse IgG1 were gifts from Professor Adrian Harris, Institute of Molecular Medicine, University of Oxford, UK.

iii. Mouse monoclonal anti-CA IX M75 IgG2b was a gift from Professor Jaro Pastorek Institute of Virology, Slovak Academy of Sciences, Slovak Republic.

iv. Endothelial marker mouse monoclonal anti-CD31 clone JC/70A IgG1 kappa purchased from Dako, Ely, Cambridgeshire, UK, Ely, Cambridgeshire, UK.

v. Endothelial marker mouse monoclonal anti-CD34 cloneQBEnd/10 IgG1 purchased from Novocastra, Newcastle Upon Tyne, UK.

vi. Fibroblast marker mouse monoclonal anti-vimentin clone V9 mouse IgG1 purchased from Dako, Ely, Cambridgeshire, UK.

vii. Fibroblast marker mouse monoclonal anti-smooth muscle actin (SMA) clone 1A4 IgG2a purchased from Dako, Ely, Cambridgeshire, UK.

viii. Macrophage marker mouse monoclonal anti-CD68 clone PGM-1 IgG3 purchased from Dako, Ely, Cambridgeshire, UK.

ix. Mouse monoclonal anti-pEGFR (Mab3052) purchased from Chemicon, Harrow, UK.

x. Rabbit polyclonal anti-phospho (p)Akt Ser^{473} purchased from Cell Signaling, MA, USA.

xi. Rabbit monoclonal anti-α tubulin purchased from Dako, Ely, Cambridgeshire, UK.

2.1.4. Secondary antibodies

i. Rabbit anti-mouse conjugated horseradish peroxidase (HRP) purchased from Amersham Pharmacia, Amersham, UK.

ii. Donkey anti-rabbit conjugated HRP purchased from Amersham Pharmacia.

iii. Goat anti-mouse conjugated HRP mainly IgG purchased from Dako, Ely, Cambridgeshire, UK.

iv. Rabbit anti-goat conjugated HRP mainly IgG purchased from Dako, Ely, Cambridgeshire, UK.

v. Rabbit anti-mouse biotinylated antibody mainly IgG purchased from Dako, Ely, Cambridgeshire, UK.

vi. Goat anti-mouse conjugated polymer antibody: mainly IgG from the Envision kit purchased from Dako, Ely, Cambridgeshire, UK.

vii. Biotinylated rabbit anti-mouse mainly IgG purchased from Dako, Ely, Cambridgeshire, UK.

2.1.6. Cell lines

A549 cells were purchased from European Collection of Animal Cell Cultures purchased from Porton Down, Wiltshire, UK.

2.1.7. Cell culture media

A549 cells were grown in Diabeco's modified eagle medium purchased from Sigma-Aldrich Company Ltd (Poole, UK) containing 10% foetal calf serum purchased from Gibco, Paisley, UK.

2.1.8. Immunohistochemistry kits

1. Envision kit purchased from Dako, Ely, Cambridgeshire, UK.
2. Catalysed signal amplification (CSA) kit purchased from Dako, Ely, Cambridgeshire, UK.
3. ABC kit purchased from Dako, Ely, Cambridgeshire, UK.

2.2. IMMUNOHISTOCHEMISTRY

IHC has been developed over the last 20 years and become a valuable tool in medical research and diagnostics. The main principle involves the application of a primary antibody to a tissue section, which detects the presence of an antigen of interest in either paraffin embedded or snap frozen tissue. Visualisation of the primary antibody-antigen complex is achieved via various techniques outlined below involving further application of a labeled secondary antibody. Good tissue preservation and accessibility of antigens are essential for reliable results.

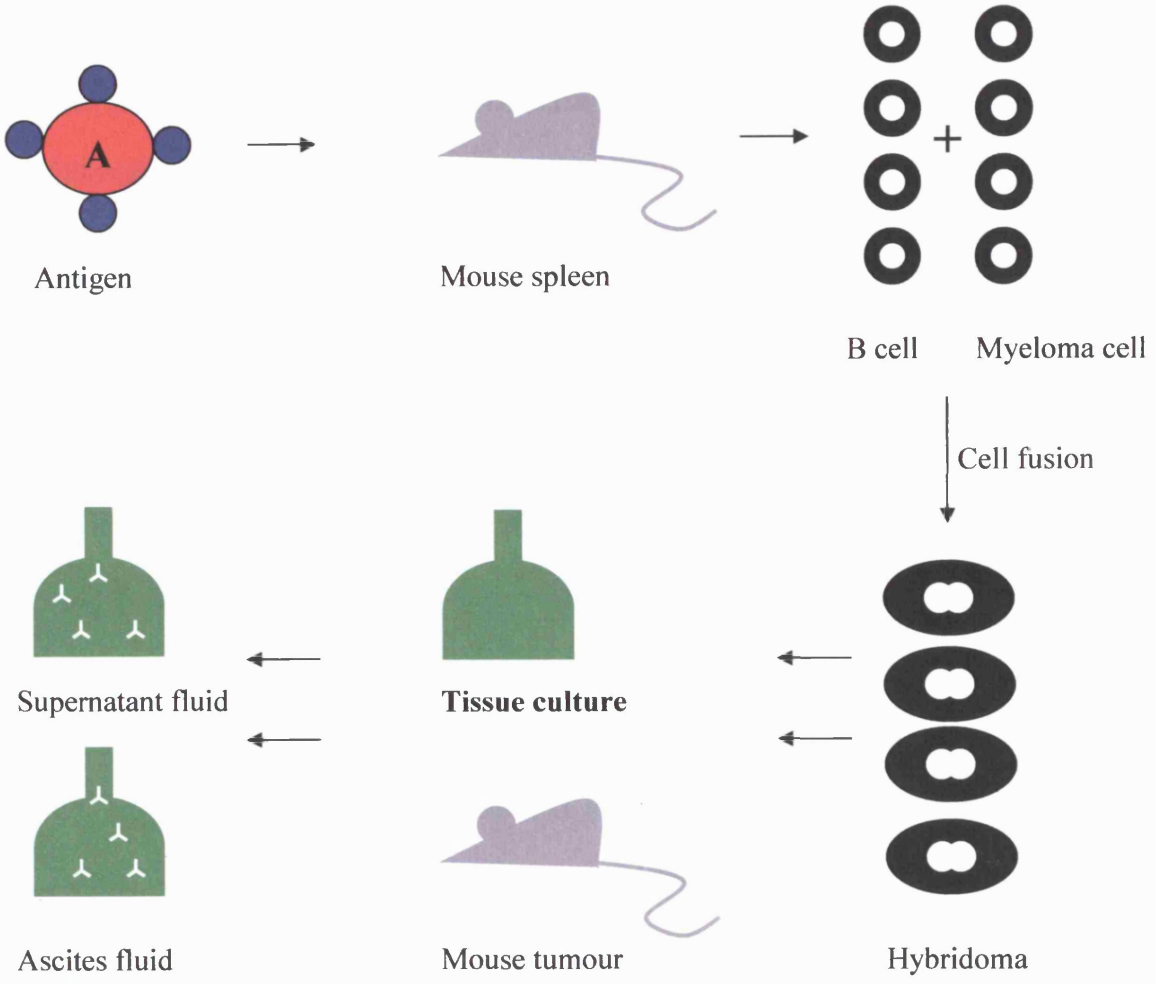
2.2.1. Antibodies

The primary antibody can either be monoclonal or polyclonal. The site on the antigen at which an anitbody binds is known as the epitope. Monoclonal antibodies recognise 1 epitope whereas polyclonal antibodies recognise different epitopes on the same antigen. As such monoclonal antibodies produce less non-specific staining, are easier to characterize and have decreased batch to batch variability. However it is important that the screening methods are similar to their intended use *i.e.* if planned to use in formalin fixed paraffin embedded tissue then formalin fixed paraffin embedded tissue should be used during screening not fixed frozen sections. Targeted epitopes must survive fixation and be able to be retrieved (see below). The targeted epitope must be unique to the antigen or else the specificity of the antibody will be lost.

2.2.2. Production of monoclonal antibodies

An immune response is initiated in a mouse by injecting the antigen of interest. B-lymphocytes are harvested and fused with myeloma cells to form a hybridoma. Hybridomas can be maintained *in vitro* for an indefinite period of time. Monoclonal antibodies are harvested by removing culture medium from the hybridoma cell lines. Alternatively the hybridomas can be grown as tumours in mice and antibodies harvested from ascitic fluid (see figure 2.1.).

Figure 2.1. Production of monoclonal antibodies.



2.2.3. The different steps of IHC

i. Dewaxing and rehydration

ii. Antigen retrieval

iii. Blocking of endogenous peroxidase

iv. Blocking of non-specific protein binding

v. Application of and viusalisation of primary antibody

vi. Mounting of slides

2.2.3.1. Dewaxing and rehydration

Sections are submerged in xylene for 5 minutes to dissolve the parafin based wax. Sections are then passed through graded alcohols 2x100% industrial methylated spirits (IMS) and 1x95% IMS, followed by 5 minute submersion in water.

2.2.3.2. Antigen retrieval

Antigen retrieval is a process that uncovers epitopes that may be masked during tissue fixation and paraffin processing. Tissue fixation in formaldehyde creates crosslinks between proteins. Paraffin processing of tissues alter the three dimensional structure of proteins. This is not a universal occurrence but it is important during optimisation studies to investigate the need for antigen retrieval and to ensure that the technique employed will fully expose the epitope of interest by uncoupling the crossbridges and reconstructing the normal 3D structure. Both heat based and proteolytic (with Trypsin) pretreatments are available and both may be required. Traditionally 0.01mol/L citrate buffer, pH 6.0 has been used as the heating buffer. Recently, both high and low pH buffers (e.g. Tris/ EDTA, pH 9.0) have been shown to improve results [348]. Several types of heat-based pre-treatments have been used in this study all of which have some benefits and problems.

2.2.3.2.1. Pressure cooking

Pressure cooking heating produces the most uniform heating methods [349]. A further advantage is that the process is relatively quick, as sections only need to be treated for 180 seconds. A pressure cooker of 103 kPa/15 psi will ensure a temperature of 120°C. Tissue sections need to be mounted on vectabond slides as otherwise these will float off during boiling; however despite these precautions tissue sections are still damaged.

2.2.3.2.2. Water bath (low temperature overnight)

A conventional water bath is able to maintain temperatures of up to 90°C providing a gentle and unifrom antigen retrieval. Prolonged incubations may be required often overnight for adequate uncovering of epitopes.

2.2.3.2.3. Water bath (high temperature 40 minutes)

A covered water bath is required to increase temperatures to near boiling and shorten the incubation time. These water baths are smaller than conventional water baths. This limits the number of slides that can be processed in 1 run, but produce excellent results.

2.2.3.2.4. Microwaving

Microwaving is an efficient method of heating aqueous solutions. There can be problems with a lack of a uniform temperature distribution across the section that results in patchy staining. Standardisation of procedures is important i.e. constant target retrieval buffer volume, constant processing time and constant number of slides to ensure an equality between different runs.

2.2.3.3. Blocking endogenous peroxidase

Endogenous peroxidase is quenched by submering sections in hydrogen peroxidase.

2.2.3.4. Blocking background staining

The primary or secondary antibodies may bind to exposed antigens other than the antigen of interest, which will generate a large degree of background staining. The affinity of the binding to these non-specific antigens is lower than the binding to the antigen that the antibody has been raised against. To minimise the background staining, sections are incubated with serum that is normally

derived from the same animal that the secondary antibody is raised in. Antibodies present in the serum compete for binding with the non-specific antigens, thereby reducing background staining. This process is not always necessary when using a monoclonal primary antibody, as the specificity is higher than that of a polyclonal antibody.

2.2.3.5. Application and visualization of primary antibody

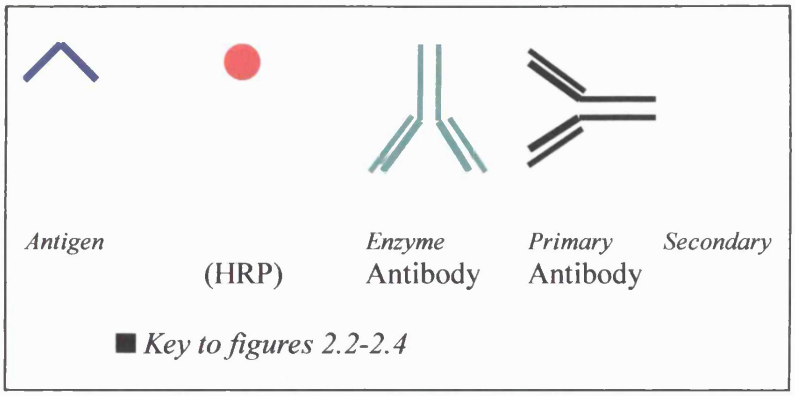
The sections are incubated with the primary antibody, which may be diluted in either a buffered solution or in the blocking serum.

The bound primary antibody is visualised by immuno-enzymatic staining methods that utilize enzyme-substrate reactions to convert a colourless chromogen into a coloured end product.

$$\text{substrate + chromagen} \xrightarrow[\text{enzyme}]{} \text{coloured end product}$$

Enzymes used in this project are horse-radish peroxidase (HRP) and calf intestine alkaline phosphatase. In the presence of H_2O_2 (substrate), HRP oxidases 3'-Diaminobenzidine tetrahydrochloride (DAB) (the chromagen) resulting in a brown end product. Alkaline phosphatase hydolyzes naphthol phosphate esters (substrate) to phenolic compounds and phosphates. The phenolic compounds couple to insoluble coloured azyo dyes (the chromagens). The azyo dye used in this project was New Fuchsin, which turns red.

A number of different techniques are available that can amplify the signal aiding interpretation. These techniques will be discussed below.



2.2.3.5.1. Direct IHC method

A HRP conjugated primary antibody reacts directly with the tissue antigen. Subsequent use of the substrate and chromagen concludes the reaction sequence. This method although rarely complicated by back ground staining achieves a poor signal and is rarely used see figure 2.2.

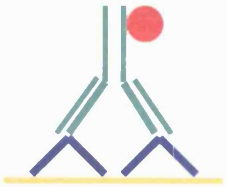


Figure 2.2. Direct IHC method

2.2.3.5.2. Two-step indirect method

In this method an unconjugated primary antibody binds to the antigen. A HRP conjugated secondary antibody directed against the primary antibody is then applied, followed by the substrate-chromagen solution. As more than one secondary antibody is able to bind to the primary a degree of signal amplification is achieved see figure 2.3.

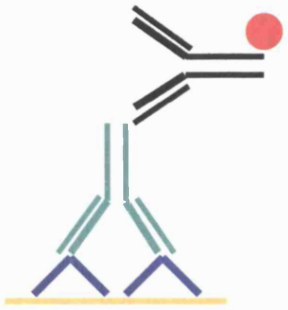


Figure 2.3. The two step indirect IHC technique

2.2.3.5.3. Three step indirect method

The initial steps are identical to the two-step indirect method but a further enzyme conjugated tertiary antibody is administered see figure 2.4.

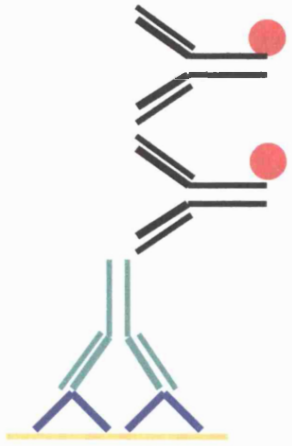
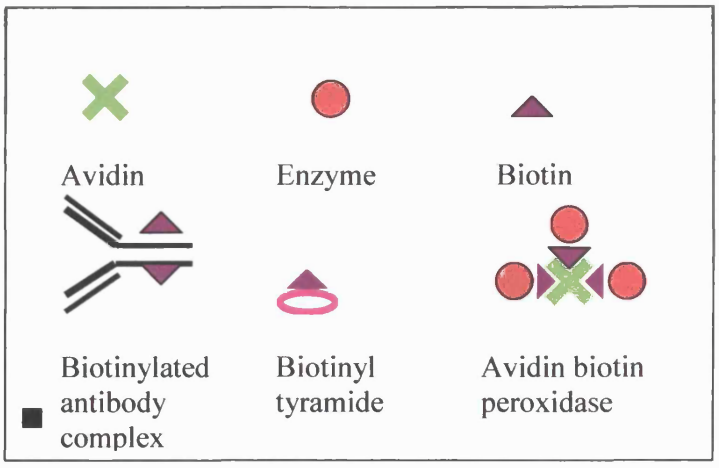


Figure 2.4. The three step indirect IHC technique



2.2.3.5.4. Avidin-Biotin Complex (ABC) method

This methods utilises the high affinity streptavdin (avidin) has for biotin. Avidin has 4 sites, to which HRP conjugated biotin can covalently bind. A biotinylated secondary antibody is administered followed by the avidin biotin-HRP complex. The avidin biotin complexes are able to bind to the secondary antibody and to each other thus amplifying the signal to a greater degree than the aforementioned methods. Hepatic, renal and lymphoid tissue express endogenous biotin that necessitates blocking when staining these tissues as it increases non-specific staining see figure 2.5.

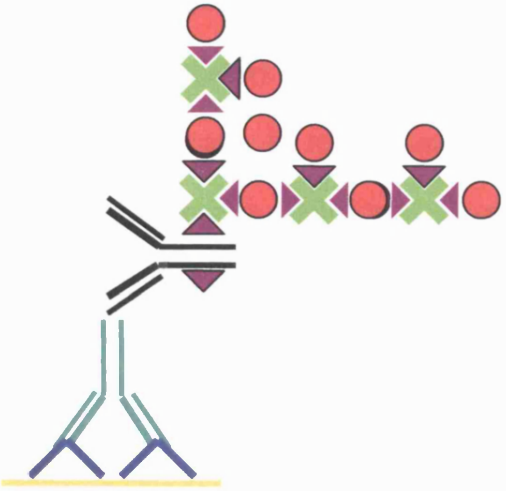


Figure 2.5. The ABC IHC method

2.2.3.5.5. Envision technique

The Envision technique involves a polymer, which is labelled with many HRP molecules and is conjugated to the secondary antibody. The polymer does not contain avidin or biotin see figure 2.6.

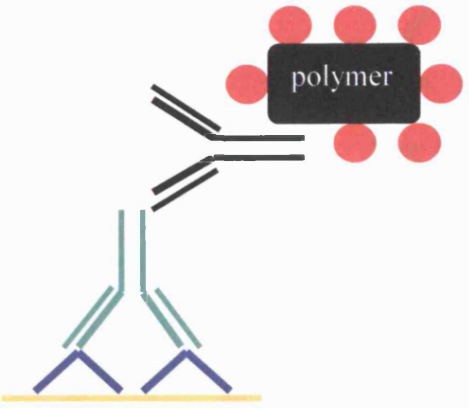


Figure 2.6. The Envision IHC technique

2.2.3.5.6. Catalysed Signal Amplification Technique

Catalyzed Signal Amplification System (CSA) is based on the peroxidase catalyzed deposition of biotinyl tyramide. A peroxidase bound secondary antibody complex is administered in 3 steps i.e. the ABC method. Biotinyl tyramide is then administered. In the presence of peroxidases, it is converted into an intermediate that is highly reactive with electron-rich aromatic amino acids found in human tissue. Hence biotinyl tyramide becomes bound to the tissue in the immediate vacinity of the of the primary-secondary antibody signal complex. HRP-conjugated streptavidin binds to the biotinyl tyramide and results in high amplification of biotin signals at the binding site of biotinylated antibodies. By using the CSA method low levels of antigen expression can be detected (figure 2.7).

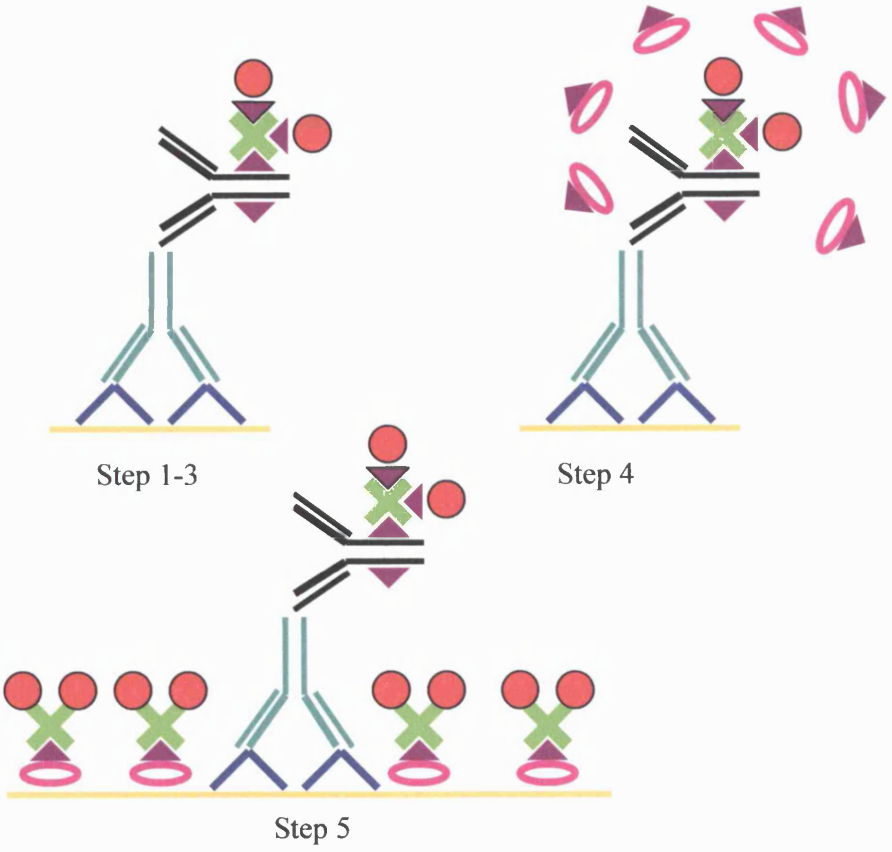


Figure 2.7. The CSA IHC technique

2.3. STUDY POPULATION

Tissue specimens were taken from 205 consecutive cases of histologically staged I-IIIA NSCLC who had undergone surgical resection with curative intent. Twenty four patients that died within 61 days of surgery were excluded from the study to reduce the confounding variable of peri-operative mortality. Further cases were excluded due to lack of available tissue, the number of which increased with successive studies. The 30-day mortality was 6.4%, which is marginally higher than internationally published 30-day mortality (3.9%) [350]. Of the 181 cases that fulfilled the inclusion criteria, 127 were male and 54 were female. The median age at surgery was 65.9 years (range 45.3). One hundred and twelve cases (61.9%) were squamous, 51 (28.2%) were adenocarcinoma, 14 (7.7%) were large cell cancers and in 4 (2.2%) subclassification was not possible. Eighty eight cases (48.6%) were stage 1, 51 (28.2%) were stage 2 and 42 (23.2) were stage 3A. Ninety three (51.4%)

cases were well to moderately differentiated and 88 (48.6%) were poorly differentiated. Positive resection margins were found in 16 cases (8.4%), the presence of which was associated with stage 3A disease (n=8/16, p=0.023). One case received adjuvant chemotherapy and 15 cases were treated with adjuvant radiotherapy of which 10 had stage 3A, 6 stage 2 and 1 stage 1 disease. The final staging was based on the findings at surgery and the histopathology report. Hospital notes of the patients were reviewed and if necessary the local cancer registry or patient's General Practioner were contacted to complete case follow up. From this data it was established that 137 (75.7%) patients had died at the time of analysis and of these deaths 18 were not cancer related.

2.4. TISSUE PREPARATION

2.4.1. Paraffin-embedded formalin-fixed tissue.

After tissue was resected it was fixed in formalin for 24-72 hours, prior to review by a histopathologist. Selected tissue blocks were processed in a standard automated fashion at 53-56°C and then embedded in paraffin wax. Blocks were kept in the dark at 4°C prior to sectioning.

2.4.2. Tissue sectioning

All IHC was performed on 4μm thick paraffin embedded tissue sections. Blocks were placed on ice for 30 minutes and cut using a microtome onto glass slides that had previously been treated with 2% 3-aminopropylethoxysilane (in methonal). Slides were dried and kept overnight at 37°C to improve section to slide adhesion. Sections were stored in the dark at 4°C until used. Previous studies by our group have shown no deleterious effect with regard to the immunoreactivity of slides stored in this manner for up to 1 year.

2.5. ANTIBODY STORAGE

On arrival antibodies H1α67, anti CD34 and anti CD31 were split into 5-10μl vails and stored at –20°C. Antibodies M75, ESEE, E190b and the secondary antibodies were stored at 4°C.

2.6. SUMMARY OF IMMUNOHISTOCHEMICAL TECHNIQUES (table 2.1)

Table 2.1. IHC techniques used

Antibody	Manufacturer	Cell type marker	Antigen Retrieval	Primary Antibody dilution	Visualisation technique
Smooth muscle actin	Dako	Activated fibroblast	Nil	1:400	ABC +DAB
Vimentin	Dako	Fibroblast	0.1% Trypsin 10 minutes at 37°C	1:50	ABC +DAB
CD34	Novacastra	Endothelial cell	Nil	1:50	ABC+DAB
CD34 Double staining	Novacastra	Endothelial cell	Nil	1:50	APAAP+ Fuscin
CD31	Dako	Endothelial cell	0.1% Trypsin 10mins at 37°C	1:50	ABC+DAB
Anti-HIF-1α clone H1α67	Novus	n/a	40 minutes in Dako antigen retrieval buffer at 96°C	1:1000	CSA kit
Anti-HIF-2α clone EP190b	Gift see section 2.1	n/a	Pressure cook 3 mins in pH 6.0 citric acid	neat	2-step indirect method
Anti-CA IX clone M75	Gift see section 2.1	n/a	none	1:50	Envision kit

2.7. INTERPRETATION

2.7.1. Interpretation of tumour necrosis

Sections were assessed at low magnification (x40) using light microscopy by two observers independently in a blinded fashion. Where a disagreement was recorded a consensus was determined using a dual headed microscope. JLJ, a consultant histopathologist, adjudicated unresolved differences. The degree of tissue necrosis (TN) on the section was scored 0=no necrosis; 1= 1 focus of necrosis per low power field (LPF), each occupying <10% of the field; 2= >1 focus of necrosis per LPF each occupying <10% of the field or 1 focus of necrosis per LPF occupying 10% - <30% of the field; 3= single or multiple areas of necrosis per LPF each occupying >30% of the field. A mean of 10 LPF per section were evaluated depending on the size of the section (figures 2.9-11). The median score was used as the cut point, which defined scores $\geq$3 as extensive and scores <3 as negative or limited.

Reproducibility of interpretation was tested 4 months after initial interpretation by randomly selecting and re-interpreting 40 cases. Agreement was found in 36/40 cases, which gave a Kappa score of 0.8 $p<0.001$.

Figure 2.8a-b: Haematoxyllin and eosin sections of NSCLC. Arrows identify necrosis. (2.8a) Large arrow: ≥30% focus of necrosis per low power field (LPF); (2.8b) Small arrow: <10% focus of necrosis per LPF; large arrow: 10% to >30% focus of necrosis per LPF.

Figure 2.8a.

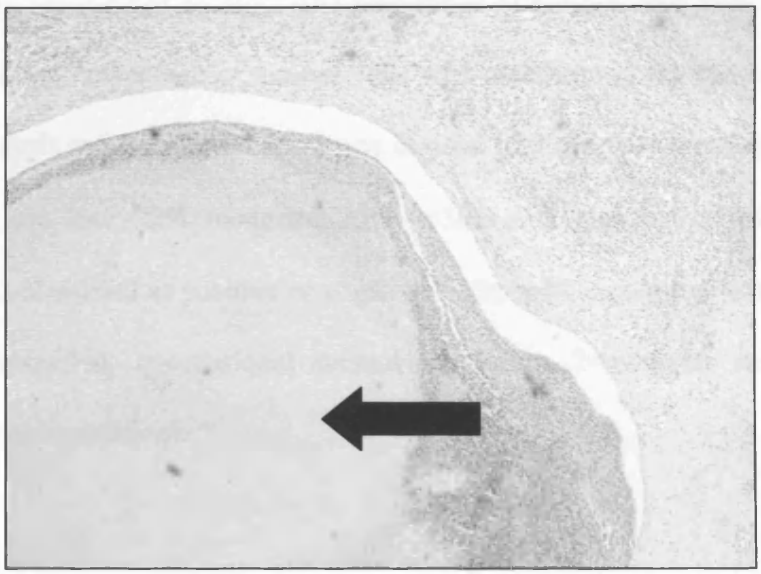
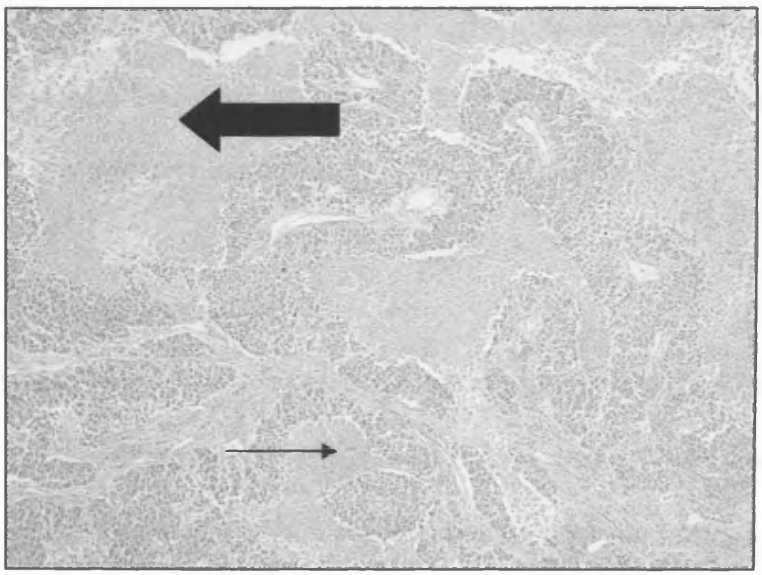


Figure 2.8b.



2.7.2. Interpretation of CA IX expression

Sections were assessed using light microscopy in a blinded fashion by 2 observers. Where discrepancies were found a consensus was reached, using a conference microscope. Persistent discrepancies were adjudicated by JLJ. The percentage of tumour cells with membranous (m) CA IX expression and the percentage of tumour cells with cytoplasmic (c) expression were estimated. For subgroup analysis mCA IX expression was divided into quartiles depending on the % of cells stained: negative: 0%, low: <5%, moderate: ≥5% - <30% and extensive: ≥30%. Perinuclear (p) CA IX expression was classified as positive or negative. Stromal expression was assigned a score 0-3 (0=no stromal expression, 1=occasional stromal expression, 2=moderate stromal expression and 3=extensive stromal expression).

2.7.3. Interpretation of HIF-1α staining

Sections were assessed in a similar manner. Above or equal to 5% staining was defined as positive expression and above or equal to 60% was defined as high expression. Reproducibility of interpretation was assessed by randomly selecting 20 slides, which were re-interpreted by JLJ and myself. Agreement was achieved in each case.

2.7.4. Interpretation of HIF-2α staining

The initial method of interpretation involved viewing the slides through a graticule that had a grid etched on it. The number of clusters of HIF-2α positive macrophages was counted on each section. This figure was divided the number of grid squares that covered each section. This gave a ratio of the number of HIF-2α positive macrophages per set area of tumour tissue. Leek *et al.* described a similar technique in a study in breast cancer [151]. Due to problems encountered in the

reproducibility of staining this method was changed to simply assessing the sections as either positive or negative with regard to the presence or absence of HIF-2α positive macrophages.

2.8. RANDOM SELECTION OF CELLS AND THE MEASUREMENT OF THE DISTANCE TO THE NEAREST MICROVESSEL VESSEL

A method was devised to compare cell to blood vessel distances between membranous (m)CA IX positive and negative tumour cells. This method was also used to measure distances from tumour cells within areas of high and low mCA IX expression, regardless of the mCA IX status of the tumour cell identified.

Case id numbers were randomly selected by using the Microsoft Excel random number generator. Randomly selected tissue sections were double stained for CA IX and the endothelial cell marker CD 34. Three areas from each case that encompassed tumour nests and blood vessels were photographed and printed onto A4 paper. To randomly identify tumour cells within these areas the centre of the photographs were overlaid by a 10 by 6 square grid (figure 2.9). Sixteen squares were randomly selected again using the Microsoft Excel software. If a tumour cell was at the centre of a selected square in the grid the distance to the nearest blood vessel was measured and the presence of tumour cell mCA IX staining was noted. After a total of 4 measurements were taken no further measurements were taken from that case. If tumour cells were found at the centre of less than 4 squares no further squares were chosen and less than 4 measurements were accepted if this was the case, to avoid bias.

The measurements were grouped firstly, as either from mCA IX stained or unstained cells and as either from areas of ≥ median mCA IX staining or from areas of < median mCA IX staining.

Figure 2.9. Photograph of tissue section overlaid by a grid to select random tumour cells

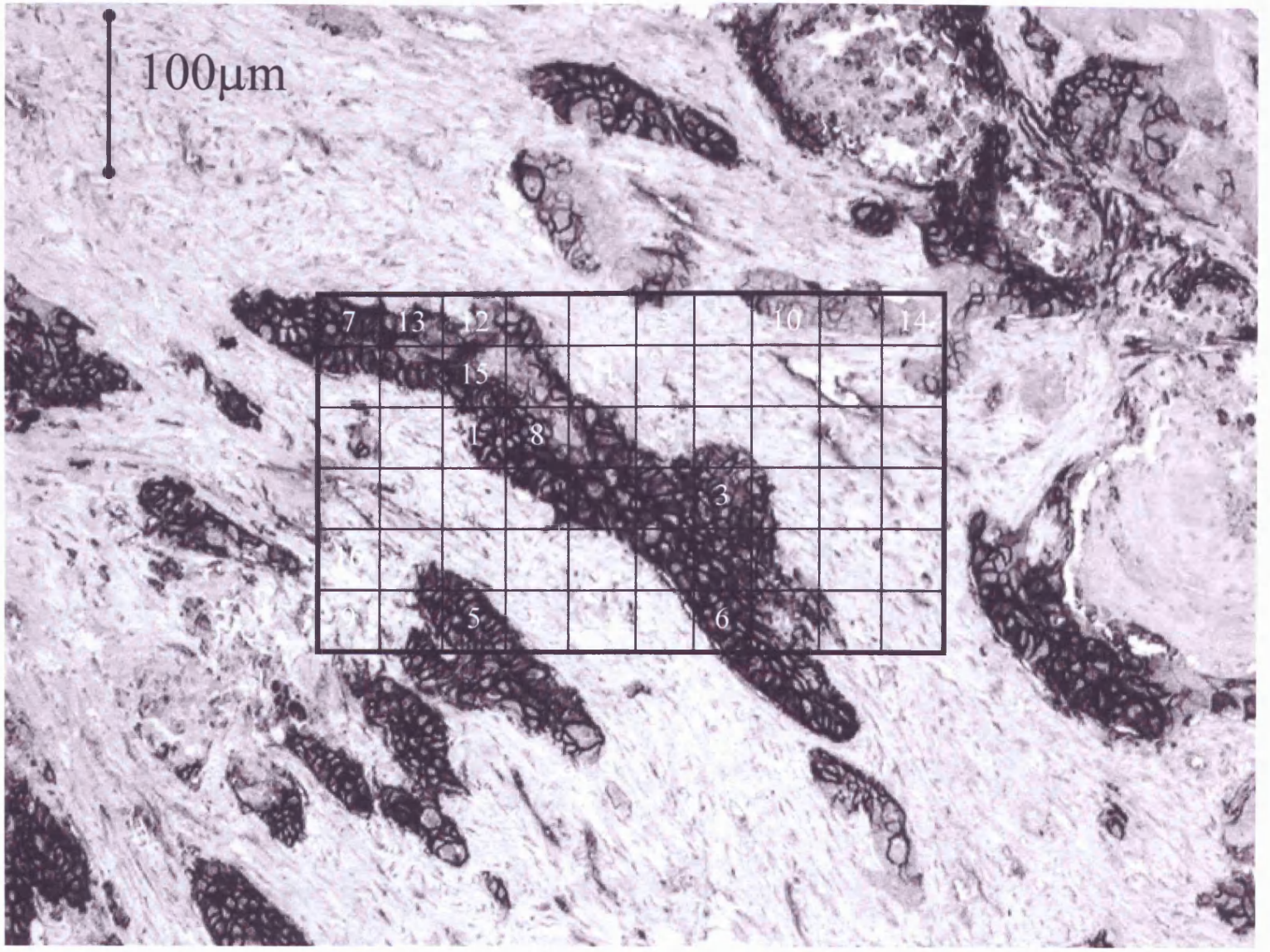
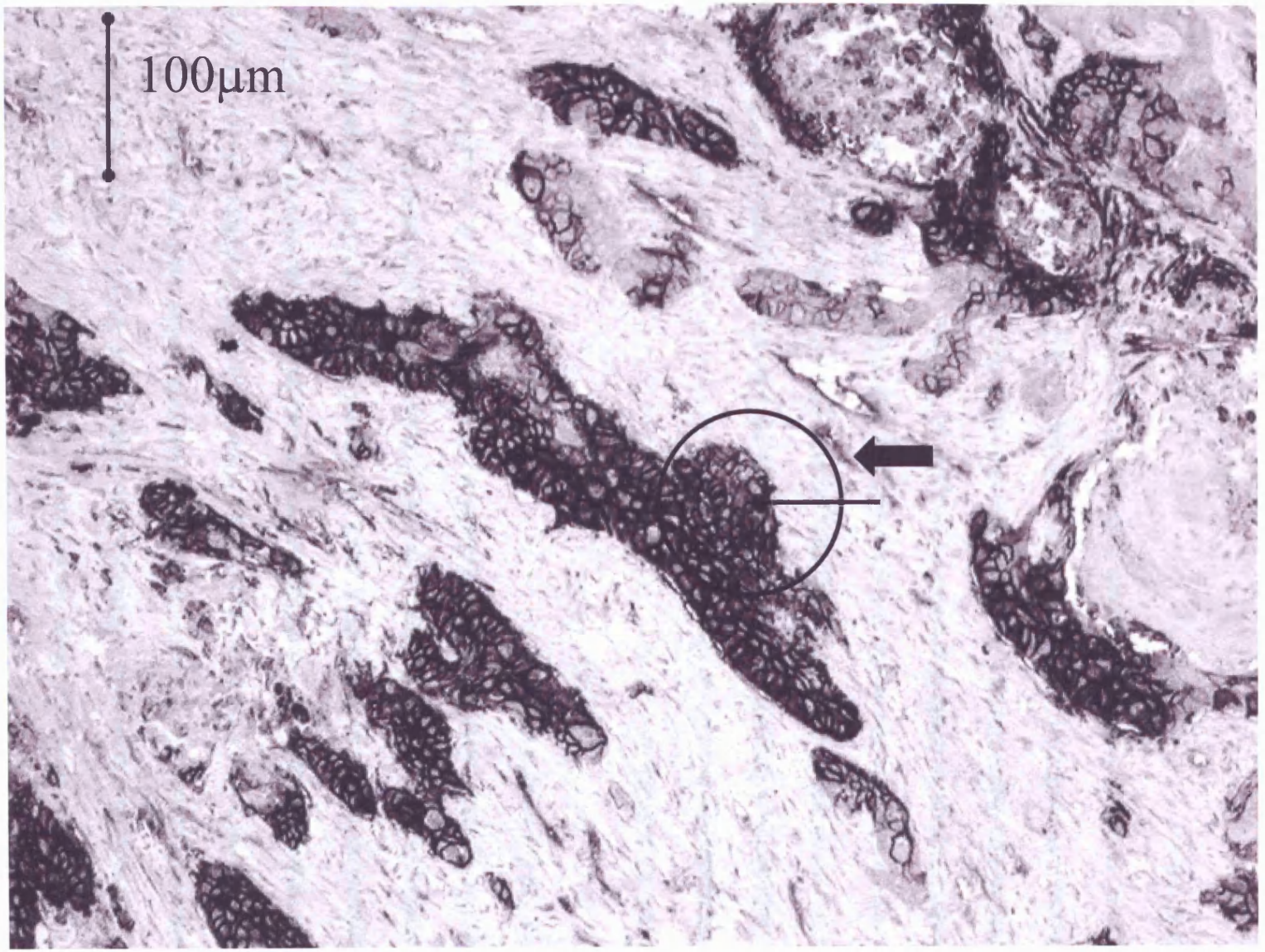


Figure 2.10. Distance from blood vessel to positive mCA IX cell



2.9. ESTIMATION OF THE pO_2 AT WHICH MEMBRANOUS CA IX STAINING STARTS

To assess at what distance from blood vessels mCA IX staining commenced 3 cases were chosen that had extensive expression. Serial sections were stained for CD 34 and CA IX, as described above. Measurements were only taken from blood vessels that were surrounded by tumour cells with positivity so that 3 distances could be made from each vessel in different directions. In addition blood vessels that had been cut through an oval or longitudinal plain were avoided. In total 6 blood vessels were suitable and 18 measurements were made (figure 2.10). The distance from the CD34 staining endothelium to mCA IX positive tumour cells was measured using a graticule with a slide wire micrometer, calibrated with a calibration slide. Beasley NJ *et al.* have previously been described this method [106].

2.10. CELL CULTURE

2.10.1. Resuscitation from liquid nitrogen

Cells retrieved from a liquid nitrogen dewer were placed in an incubator at $37^{o}C/5\%CO_2$ until the vial had completely thawed. They were then added to 5ml of warmed media in a $25cm^2$ flask. After approximately 8 hrs the media was removed and replaced with fresh media so as to eradicate dimethyl sulphoxide (DMSO) present in the cryomix. The cell line used was tested for mycoplasma contamination (Mycoplasma Experience, UK) and was found to be free of any infection. Following attainment of confluency, cells were passaged as below to a $75cm^2$ and then to a $150cm^2$ flask. All reagents and equipment used in these experiments were prepared and kept under strictly aseptic conditions.

2.10.2. Routine splitting of flasks and subculture of cell lines

Once cells had reached confluency in 150cm^2 flasks they were passaged. 5ml of trypsin/EDTA (T/E) solution was used to wash the cells following removal of the medium. A further 5ml of T/E solution was used to detach the cells from the base of the flask. Usually 5 mins at 37°C was sufficient. Following this the cell suspension was added to an equal volume of warmed media in a universal and spun for 5 mins at 300g. The cell pellet was then resuspended in fresh medium, and the resultant suspension used to seed 90mm or 140mm cell culture plates for experimentation, usually 1:10 or 1:20 dilution. Two millilitres of the remaining cell solution was added to 12ml of media in a 150cm^2 flask and re-incubated to grow to confluency.

2.10.3. Treatment and harvesting of cells

Cells were grown in either 90mm or 140mm dishes. The media was removed and the cells were briefly washed with warmed PBS. The cells were then incubated in serum free media for 0-48 hours. After administration and incubation with the allotted test reagents cells were harvested. Culture media was removed and cells were rinsed 3 times in PBS. After each rinse the PBS was sucked off. Lysis buffer (200µl) was then administered and the cells and lysis buffer were removed by scraping the cell culture dish with a pallet knife. The cells were placed in ependorff tubes and left on ice for 20 minutes. Samples were spun at 3000rpm for 15 minutes to separate out the clean supernatant solution from cellular debris. Two hundred microlitres of the supernatant was added to 200µl of 2x sample buffer. Samples could either be stored at –20°C before or after mixing with sample buffer. Samples were then ready to be loaded into gels after heating for at 100°C for 5 minutes.

2.11. WESTERN BLOTTING

2.11.1. Biorad Protein concentration assay

For accurate Western blotting results it is essential that a uniform quantity of protein be run in each lane of the gel. The biorad protein assay calculates the protein concentration of the lysate solution therefore allowing calculation of the volume of lysate required for individual samples to be added to each lane of the gel. The first step was to construct a standardised curve using a series of solutions containing known protein concentrations. The range of concentrations formulated and the requisite volumes of a 1mg/ml bovine serum albumin (BSA) standard plus water to reach the desired concentration in a 1ml cuvette are shown in table 2.2.

Protein (mg/ml)	BSA standard (µl)	water (µl)
0	0	800
2	1.6	798.4
4	3.2	796.8
6	4.8	795.2
8	6.4	793.6
10	8.0	792.0
12	9.6	790.4

Table 2.2. Volumes to make standard BSA solutions

Biorad reagent (200µl) was added to each of the defined solutions and mixed by simple inversion with parafilm. Each concentration was duplicated to reduce inter-sample error, the absorbance at 595nm was read on a spectrophotometer using 0mg/ml BSA as a blank and the average reading from

the duplicated samples was plotted against the known concentrations to make a standard curve. Subsequently 4µl of each sample was added to 796µl water and 200µl of Biorad reagent and mixed in a 1ml cuvette. Again, each sample was duplicated and the absorbance read. Using the standardised curve the concentrations of each sample were calculated. This method makes the assumption that the degree of Biorad reagent binding does not differ between BSA and the sample protein.

2.11.2. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE separates proteins according to their molecular weight. An electric field through the gel induces proteins to traverse the gel at differing rates according to their molecular weight. An increase in the molecular weight or percentage of acrylamide in the gel will inhibit the rate at which a protein traverses a gel. The range of acrylamide concentrations within a gel are therefore varied in accordance with the molecular weight of the protein under investigation. A gel consists of a stacking and a resolving gel. A 5% stacking and 10% resolving gel was used for CA IX and pAkt experiments, the standard solutions for which are listed below in table 2.3.

	10%	5% (stack)
H_20	5.9ml	3.4
30% w/v acrylamide and bis-acrylamide	5.0ml	0.83
1.5M Tris-HCl, pH8.8	3.8ml	-
0.5M Tris-HCl, pH 6.8	-	0.63
10% SDS	0.15ml	0.05
10% ammonium persulphate	0.15ml	0.05
N,N,N',N'-tetramethylenediamine (TEMED)	0.006ml	0.005

Table 2.3 Recipes for SDS-PAGE

2.11.3. Gel pouring

The Biorad mini protean III kit was used for SDS-PAGE and was assembled according to the manufacturer's instructions. Prior to gel pouring the apparatus was partially filled with water to check for leaks. The lower gel reagents were mixed in a 50ml universal container. TEMED was added immediately prior to pouring as it initiates the polymerisation reaction. A thin layer of water was floated over the gel as it sets to prevent air bubbles, thereby ensuring a level gel surface. After

polymerisation (approximately 30 mins) the water was poured off, the stacking gel was added and a 10-tooth comb inserted. The comb was removed once the stacking gel had set.

2.11.4. Gel running, Western blotting and protein transfer

The inner electrode kit was assembled and filled with ~100ml of running buffer above the level of the stacking gel wells. The left hand lane of the stacking gel was filled with 5µl of biorad kaleidoscope markers. These are molecular weight markers. The calculated volumes of samples were added to the adjacent wells, using rounded gel loading pipette tips. The assembled kit was placed in a gel tank along with the remaing 400ml of running buffer. The power pack electrodes were attached to the gel tank lid and the power pack was set at 150V. The dye front could be visualised traversing downwards through the gel and was run until it was off the bottom. The gels were then removed from the electrode kit. The Biorad wet blotting apparatus (transfer kit) was assembled in accordance with manufacturers instructions (figure 2.11.). Prior to assembly the nitrocellulose membranes, sponge and blotting paper were soaked in transfer buffer for an hour, 15 minutes and 15 minutes, respectively. The transfer kit was then placed in the tank, which was filled with transfer buffer to above the level of the gel. Protein transfer from the gel to the nitrocellulose membrane could be performed over night at room temperature with the power pack set at 30V or at 4°C for 90 minutes with the power pack set at 100V (Western blotting).

Figure 2.11. Assembly of wet blotting apparatus

2.11.6. Detection of proteins

Following transfer, membranes were blocked for 1 hour at room temperature in 10% marvel TBS-T. Blots were then rinsed in TBS-T and incubated with the primary antibody, overnight at 4°C. Unbound primary antibody solution was removed by 3x5-min submersions in TBS-T. Blots were then incubated in HRP-conjugated secondary antibody for 45-60 mins at room temperature. Anti-mouse secondary antibodies were diluted in TBS-T/5% marvel. Again unbound secondary antibody was removed by 3x5-min submersions in TBS-T. Blots were finally submerged in 1.8ml of enhanced chemiluminescene (ECL) reagent (Amersham Pharmacia) for less than a minute. The blot was developed under safelight conditions, using Hyperfilm (Amersham Pharmacia) after excess reagent was removed.

2.11.7. Stripping

To check that an equal amount of protein had been loaded into each lane, the primary and secondary antibodies were stripped off the blots. The bare blots were then re-probed for a housekeeper protein. A housekeeper protein is one that is produced by all cells in uniform amounts under different conditions. The housekeeper protein used was α-tubulin.

The process entailed blots being submerged in 50ml of stripping buffer for 45 minutes at 60°C in an incubator. The blots were then rinsed 3x in TBS-T, incubated in TBS-T for 30 minutes and then further 3x 5 minute washes in TBS-T. The primary antibody against the housekeeper protein was applied at 4°C overnight and the blots developed as previously described.

2.12. STATISTICS

The SPSS software system (SPSS for Windows Version 9.0, www.SPSS.com) was used to perform the statistical analysis as previously described [197]. The χ^2-test was used to analyze the associations between categorical variables. The statistical significance of trends between the increasing values of different categorical variables was analysed, using the linear-by-linear association test. Survival curves were plotted using the Kaplan-Meier method and the statistical significance was assessed using the log-rank test. A Cox proportional hazards regression model was used, to identify statistically significant differences in survival and estimate hazard ratios. Ninety five percent confidence intervals were used. The assumption of proportional hazards was assessed graphically by plotting log-minus-log of survival against time for each of the prognostic groups. Prognostic variables identified by univariate analysis with $p<0.1$, were entered into a Cox multivariate analysis model. A forward, stepwise selection procedure was used, with variables being added to the model according to a partial likelihood ratio test, using an entry criterion of $p<0.05$. Statistical advice was sought from Dr. N. Taub.

Chapter 3

Tumour Necrosis in NSCLC

3.1. INTRODUCTION

Hypoxia can induce cell death by 2 separate mechanisms, apoptosis or necrosis. Apoptosis is a physiologically normal event often referred to as programmed cell death, which requires energy. Hypoxia induced apoptosis has been discussed in section 1.5. Necrotic cell death is a pathological process following cellular injury often involving a solid mass of tissue and does not involve energy expenditure. There are several different types of tissue necrosis that are caused by different cellular insults and are summarised in table 3.1. Coagulative necrosis is the type most frequently observed in tumours where it is commonly caused by diffusion-limited hypoxia. Elsewhere coagulative necrosis is caused by acute ischaemia, for example following a myocardial infarction. The appearance of this type of necrosis depends on the time from which the tissue concerned has been devitalised. Initially cells retain their outlines and the texture of the tissue remains firm as intracellular proteins coagulate and metabolic activity ceases. Subsequently there is progressive loss of nuclear staining until it ceases to be haematoxyphillic; this is accompanied by loss of cytoplasmic detail. The collagenous stroma is more resistant to dissolution with the result being that tissue retains a faint outline of its structure until it is removed by phagocytosis [351, 352].

Table 3.1. Causes and types of tissue necrosis

Type of necrosis	Cause and brief description
Coagulative	See text
Colliquative	Necrosis seen in the brain, due to lack of supporting structure tissue is liable to liquifaction.
Caseous	Tuberculosis is characterised by caseous necrosis but there are other causes.
Gangrene	Putrefaction of tissues caused by some bacteria i.e. clostridia appears black.
Fibrinoid	Seen in the context of malignant hypertension, necrosis of smooth muscle of the arterioles due to increased pressure.
Fat necrosis	Caused by direct trauma to adipose tissue or leakage of lipases during pancreatitis.

Gray et al. reported that lung cancers grew as cords around blood vessels and that tumour cells more than 180μm from the vessel underwent necrosis. This distance represents the diffusion limit of O_2 hence generating the term diffusion limited hypoxia [353]. Cell proliferation markers in breast cancer have been found to correlate with the presence of TN [354]. These observations suggest that tumour cells undergoing uncontrolled proliferation outgrow their blood supply, become hypoxic and undergo necrosis. In support of this, exposure of cultured cells to hypoxic conditions results in necrotic change [355, 356]. Acute ischaemic hypoxia also occurs in tumours as a result of aberrant vessels where the blood flow shuts down or even reverses due to the lack of the normal circulatory controls i.e. vascular smooth muscle and neural innervation [113, 357]. These aberrations of blood flow in tumours may persist inducing necrotic change or be temporary with rapid re-oxygenation of

the affected tissue. In the event of re-oxygenation necrosis can still occur, as this process can result in the release of free radicals and that cause tissue damage and necrosis [358]. Given that both acute and diffusion limited hypoxia are the major causes of necrosis in tumour tissue the extent of tumour necrosis (TN) may be used as a surrogate marker of hypoxia. As such TN has been shown to correlate with hypoxic recordings taken with poloragraphic probes, although this is not a consistent finding [359-362]. In addition, the presence of necrosis on histological sections correlates with the presence of increased staining of hypoxic markers [106].

TN is a recognised prognostic marker in breast cancer. There are over 20 separate studies in ductal carcinoma in situ (DCIS) and invasive breast cancer with or without lymph node involvement reporting an association between the extent of TN and decreased disease free and/ or overall survival [363-377]. Two large studies show that this association is strongest within the first 2-3 post-operative years [369, 371]. In addition to survival, TN correlates with poor prognostic indices such as high grade, increased tumour size and the absence of oestrogen receptor expression [377, 378]. In NSCLC a study of 593 surgically resected tumours found an association with poor outcome [379]. Studies in gastrointestinal stromal tumours, renal carcinoma, glioblastoma, soft tissue and bone sarcoma including Ewing's type sarcoma have found similar results [360, 380-383].

The purpose of this study was to evaluate the impact of TN on patient outcome in stage I-IIIA resected NSCLC. In addition, the relationship between TN and clinicopathological and biological parameters was evaluated.

3.2. METHODS

For tissue preparation see section 2.4.

For study population see section 2.3.

For interpretation of TN sections 2.7.

For statistics see section 2.12.

3.3. PREVIOUS IMMUNOHISTOCHEMISTRY

The specimens had previously been evaluated using standard immunohistochemistry techniques for the expression of EGFR, MMP-9, MMP-2, Bcl-2, p53 and mean vessel density (MVD) (table 3.2) [64, 197, 384]. The cut points used to define categorical variables as either high or low expression are also listed. MVD was assessed using the Chalkley counting method (n=176) [197].

Table 3.2. Previous immunohistochemical staining performed on this series.

Marker	Antigen Retrieval	Primary antibody	Secondary antibody	IHC kit	Cut point	Number Stained
EGFR	P/c 3 minutes pH 6.0	Novocastra anti-EGFR Mab Clone EGFR.113	Rabbit anti-mouse	ABC	20%	179
MMP-9	P/c 3 minutes pH 6.0	Chemicon anti-MMP-9 Mab Clone 56-2A4	Rabbit anti-mouse	ABC	20%	169
MMP-2	P/c 3 minutes pH 6.0	Chemicon anti-MMP-2 Mab Clone 42-5D11	Rabbit anti-mouse	ABC	Positive or negative	170
Bcl-2	P/c 3 minutes pH 6.0	Dako anti-Bcl-2 Mab Clone 124	Rabbit anti-mouse	ABC	20%	180
P53	P/c 3 minutes pH 6.0	Novocastra anti-p53 polyclonal Clone p53-CMI	Swine anti-rabbit	ABC	20%	180
CD34	Nil	Novocastra QB End/10	Rabbit anti-mouse	ABC	Median	177

Abbreviations: ABC: Avidin-biotin complex; MMP: matrix-metalloproteinase; EGFR: epidermal growth factor receptor; Mab: Monoclonal antibody; P/c: pressure cooking.

3.4. RESULTS

3.4.1. Associations with clinicopathological variables

TN scores above 2 were found in 76 cases and defined as extensive TN. TN was associated with T-stage ($p<0.001$), platelet count ($p=0.006$) and a non-significant association was found with positive resection margins ($p=0.082$). No association was found with N-stage ($p=0.13$, overall stage ($p=0.16$, histology ($p=0.19$), grade ($p=0.99$) gender ($p=0.82$) or age ($p=0.33$).

3.4.2. Associations with biological variables

TN was associated with p53 expression ($p = 0.042$) and a trend towards an association was found with MMP-9 expression ($p=0.071$). No associations were found with EGFR ($p=0.69$), Bcl-2 ($p=0.92$), MMP-2 ($p=0.16$) expression or MVD ($p=0.53$) (table 3.3.).

3.4.3. Univariate Analysis

The survival data from previous studies was up-dated and statistical analysis using Cox regression analysis was repeated to include TN [64, 197]. The clinico-pathological variables TN ($p=0.005$), stage ($p<0.001$), gender ($p=0.048$) and the platelet count ($p=0.035$) were prognostic. Among the biological variables MMP-9 ($p=0.002$) and MVD ($p<0.001$) were prognostic (table 3.4.) (figure 3.1).

3.4.4. Subgroup Analysis in Different Tumour Stages

Extensive TN was associated with a poor prognosis in early disease only (stages I and II) ($p=0.0014$, $n=138$). In stage IIIA cases there was no association between TN and prognosis ($p=0.54$, $n=40$).

Table 3.3. Relationships between clinicopathological, biological variables and TN

Prognostic Factor		Low TN	High TN	χ^2 p value
Age	< median	56	35	0.33
	≥ median	49	41	
Gender	Female	32	22	0.82
	Male	73	54	
Histology	Adenocarcinoma	33	18	0.19
	Large	8	6	
	Squamous	60	52	
	Other	4	0	
Grade (Differentiation)	Well/moderate	54	39	0.99
	Poor	51	37	
T - Stage	1	28	4	<0.001
	2	69	59	
	3	8	13	
N - Stage	0	60	35	0.13
	1	25	30	
	2	19	11	
Overall stage	1	56	32	0.16
	2	24	27	
	3	25	17	
Platelet Count	< median	60	26	0.006
	≥ median	44	45	
Resection margins	Negative	99	66	0.082
	Positive	6	10	
EGFR	< 20%	51	35	0.69
	≥ 20%	53	41	
MMP-9	< 20%	51	29	0.071
	≥ 20%	45	45	
MMP-2	Negative	62	40	0.16
	Positive	26	27	
Bcl-2	Positive	63	45	0.92
	Negative	42	31	
p53	<20%	63	342	0.042
	≥20%	42	42	
MVD	< median	51	38	0.53
	≥ median	51	37	

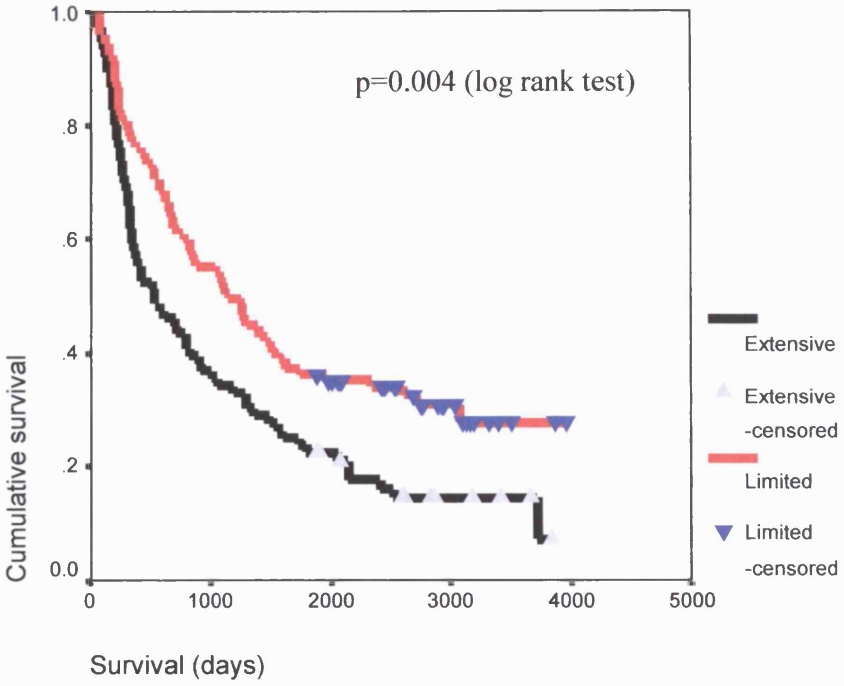


Figure 3.1. Kaplan Meier survival curve for TN in NSCLC

Table 3.4. Revised univariate analysis of clinicopathological and biological variables including TN

Prognostic Factor		n	Hazard Ratio	95% CI	p
Age (yrs)	< Median	91	1.0		0.67
	≥ Median	90	1.07	0.77-1.51	
Gender	Female	54	1.0		0.048
	Male	127	1.56	1.05 – 2.29	
Histology	Adenocarcinoma	51	1.0		0.33
	Large	14	0.84	0.39-1.82	
	Squamous	112	1.31	0.88-1.93	
	Other	4	1.8	0.64-5.06	
Grade	Well/mod diff	93	1.0		0.56
	Poor diff	88	1.1	0.79-1.54	
TNM Stage	I	88	1.0		<0.001
	II	51	1.83	1.22–2.72	
	IIIA	42	2.63	1.74-3.99	
Positive resections margins	Negative	165	1.0		0.1
	Positive	16	1.57	0.91-2.68	
Platelet Count	< median	86	1.0		0.035
	≥ median	89	1.45	1.03–2.05	
TN	Focal	105	1		0.005
	Extensive	76	1.63	1.16–2.28	
MVD	<Median	89	1.0		
	≥Median	88	1.86	1.32-2.63	<0.001
MMP-9	<20%	80	1		
	≥20%	90	1.78	1.23-2.50	0.002
Bcl-2	Negative	108			
	Positive	73			0.56
p53	<20%	97			
	≥20%	84			0.50
EGFR	<20%	86			
	≥20%	94			0.94
MMP-2	<20%	114			0.96
	≥20%	56			

3.4.5. Multivariate Analysis

All factors listed in table 3.4. that had a p value ≤ 0.1 were included in multivariate analysis. Stage (p=0.005), MVD (p=0.001), gender (p=0.009), MMP-9 expression (p=0.015), platelet count (p=0.036) and TN (p=0.05) were independent prognostic markers (table 3.5).

Table 3.5. Revised multivariate analysis of clinicopathological and biological variables including TN

Factor	Variable	Hazard Ratio	95% CI	p-value
Stage	1	1		
	2	1.568	1.008-2.439	
	3A	1.920	1.212-3.042	0.005
MVD	<median	1		
	≥median	1.858	1.278-2.70	0.001
Gender	Female	1		
	Male	1.811	1.161-2.824	0.009
MMP-9	<20%	1		
	≥20%	1.598	1.097-2.329	0.015
Platelet count	<median	1		
	≥median	1.486	1.026-2.150	0.036
TN	Limited	1		
	Extensive	1.450	1.001-2.101	0.05

3.5. DISCUSSION

These results demonstrate that extensive TN is a univariate poor prognostic factor and in multivariate analysis is an independent prognostic factor. Other independent poor prognostic factors are advanced stage, high platelet count, male gender, high MMP-9 expression and high MVD. Survival data from previous immunohistochemical studies in our series was updated; increased MMP-9 expression, MVD and platelet count retained significant associations with a poor prognosis but the previously reported prognostic significance of Bcl-2 expression was lost [64, 197, 384].

The prognostic significance of TN suggests a paradoxical relationship whereby evidence of increased tumour cell death indicates a more aggressive cancer. The apparent paradox may be explained by rapid tumour growth outstripping the vascular supply creating a hypoxic microenvironment and subsequently causing TN. Such a phenomena may be commonplace as TN is a poor prognostic marker in many solid tumours.

Our results are in agreement with a large study of 593 patients and 2 smaller studies of stage 1 NSCLC and non-neuroendocrine large cell carcinoma [385, 386]. Interestingly the relationship between TN and patient outcome is lost in the stage IIIA subgroup. A similar finding has previously been reported in a series of 30 patients treated with concurrent platinum based chemotherapy and radiotherapy. This may be due to the small size of both this and our patient samples or may reflect a real reduction in the discriminatory value of this marker in later stage disease [387].

In breast cancer and mesothelioma, TN has been found to correlate with MVD and in the former with increased macrophage infiltration [377, 388]. Macrophages are a common source of angiogenic growth factors such as VEGF. From these findings Leek at al. proposed that the hypoxic environment responsible for TN attracts macrophages that subsequently release angiogenic growth factors and stimulate angiogenesis. However in our study no association was found between MVD and the degree of TN. The lack of an association may reflect an important difference between

NSCLC and breast cancer, suggesting that hypoxia is not as important in stimulating angiogenesis in the former. Some important caveats exist, which may invalidate this initial interpretation. The estimation of hypoxia by detecting the presence or absence of TN in this study may not be accurate enough to detect an association between hypoxia and angiogenesis in NSCLC. For example in this study TN and MVD were not assessed on serial sections, which may mask an association, as there is high degree of variability in the pattern of angiogenesis throughout NSCLC tumours [389]. This is in contrast to breast cancer, where analysis of the MVD from single histological sections has been found to strongly correlate with whole tumour vascularity assessed by MRI angiogram [390]. TN may not always be an accurate measure of tumour hypoxia, for instance TN may be secondary to acute infarction in the absence of chronic hypoxia due to aberrant tumour blood flow. Under such circumstances the episode of hypoxia may be brief and although cause necrosis not be of sufficient duration to stimulate angiogenesis. Secondarily, hypoxia may not always result in necrosis as tumour cells have many adaptive processes to withstand hypoxia induced cell death [391]. Thereby hypoxia may stimulate angiogenesis but not cause TN.

The association with T-stage has also been observed in breast cancer and suggests that the presence of TN reflects extensive local growth of NSCLC tumours [377]. This has been demonstrated *in vivo*. Transplanted tumour studies that show a hypoxic gradient exists from the periphery of a tumour to the centre and that the degree of necrosis increases as a tumour grows [118, 392]. Interestingly a separate study has found that although TN increases with tumour growth initially, once a tumour reaches a critical size the relationship is lost [393]. This may explain the loss of the prognostic discriminatory value in stage IIIA NSCLC disease seen in our study.

A non-significant association was found between MMP-9 and TN in this study and a significant association has been reported in breast cancer [394]. MMP-9 is an endopeptidase that digests the extracellular matrix enhancing the invasive potential of cancer cells and facilitating angiogenesis;

increased expression is an independent prognostic factor in NSCLC. This association suggests that MMP-9 may be upregulated either by hypoxia or by factors released during tissue necrosis. Hypoxia has been shown to induce MMP-9 in some tumour cell lines [395, 396].

Under normal physiological conditions p53 regulates the magnitude of apoptosis in response to hypoxia (see section 1.5.) [160, 161]. Persistent hypoxia mediates selection for mutant p53 as cells expressing wild type p53 undergo programmed cell death [90]. As wild-type p53 has a short half-life immunostaining tends to detect overexpression of mutant p53 [397]. The association between p53 expression and TN may reflect both the selection of mutant p53 and the up-regulation of wild type p53 in non-genetically mutated cells. The lack of an association between p53 expression and outcome suggests a degree of heterogeneity exists in the population of tumour cells, some overexpressing wild type p53 and others expressing mutant p53. In breast cancer the presence of p53 positivity and absence of Bcl-2 correlate with TN [398, 399]. Both our observations and those of Tormanen et al. show only the relationship between p53 and TN is repeated in NSCLC [400].

The association between platelet count and TN in NSCLC may simply reflect the fact that both are seen in tumours with an aggressive phenotype [197]. However theoretically a thrombocytosis may directly cause TN. Vascular stasis, exposure of the subendothelial matrix and increased expression of platelet binding factors commonly occur in tumours and may lead to increased platelet adhesion and activation. In turn, activation of platelets can result in the formation of platelet plugs obstructing the vascular supply causing further TN [401, 402]. Reciprocally hypoxia may induce a thrombocytosis through induction of interleukin (IL)-6 [403]. IL-6 is a potent stimulator of platelet synthesis and is widely expressed in NSCLC [404, 405].

A possible criticism of the study is that the degree of TN was assessed subjectively. The high degree of reproducibility that was achieved when the specimens were interpreted 4 months apart

demonstrates the reliability of this method. Furthermore, previous published data investigating TN have used similar techniques [377].

In conclusion we have demonstrated that extensive TN is an independent poor prognostic factor in NSCLC and may represent an easily measurable parameter. This may add to the conventional staging of this tumour type. The positive correlations with T-stage and platelet count and the trend towards associations with tumour cell MMP-9 expression show that TN is associated with other parameters associated with an aggressive NSCLC phenotype.

Chapter 4

Carbonic Anhydrase IX in NSCLC

4.1. INTRODUCTION

4.1.1. Carbonic anhydrase

The first carbonic anhydrase (CA) was purified from erythrocytes in 1933 and was followed by the characterisation of several mammalian isoenzymes [406]. These enzymes are ubiquitously expressed in plants and a recent survey established that the enzyme is widely distributed among phylogenetically and physiologically diverse prokaryotes, indicating a highly important role for this enzyme in nature [407, 408].

Comparison of sequences from plant and mammalian enzymes demonstrate that they have evolved independently. They have been designated to α-, β- and γ-classes [409]. The α-class is the best described. The enzyme catalyses the reversible hydration of CO_2 (see equation 1).

$$CO_2+H_2O \longleftrightarrow HCO_3^-+H^+ \qquad \text{(Eq. 1)}$$

Despite gross structural differences, the active sites of all 3 classes function with a single zinc atom essential to the catalytic process. Kinetic studies indicate that all three classes employ a 2-step isomechanism. The first step involves a nucleophilic attack of a zinc-bound hydroxyl group on CO_2 (see equation 2).

$$Zn^{2+}\text{-}OH^- + CO_2 \longleftrightarrow Zn^{2+} + HCO_3^- \qquad \text{(Eq. 2)}$$

The second step is the regeneration of the active site by ionisation of the zinc bound water molecule and removal of a proton from the active site [409] (see equation 3)

$$Zn^{2+} + H_2O \longleftrightarrow H^+ + Zn^{2+}\text{-}OH^- \qquad \text{(Eq. 3)}$$

4.1.2. Mammalian carbonic anhydrase

The mammalian CA isoenymes are all members of the α-family and to date 12 have been identified. The sub-cellular location, kinetic properties and susceptibility to inhibition by sulphonamides vary. These isoenzymes are involved in a wide range of processes that involve buffering of the intracellular compartment, acidification of the lumen of the renal nephron and gastric juices, alveolar gas exchange, provision of HCO_3^- for carboxylation reactions in glucoenogenesis, lipid synthesis, the urea cycle and pyrimidine synthesis [410] (see table 4.1).

4.1.3. Role of carbonic anhydrase in the tumour environment

There are various different mechanisms by which CA activity may promote tumour cell survival and proliferation. HCO_3^- is required as a substrate for carbomyl synthetase enzymes involved in pyrimidine synthesis. HCO_3^- may be important for the production of non-essential amino acids utilised in both pyrimidine and purine synthesis. Also in terms of the synthesis of essential cell structures, HCO_3^- is required for the synthesis of membrane lipids. Endogenous HCO_3^- is normally sufficient under physiological conditions, but increased tumour cell proliferation exceeds endogenous supplies [410].

A further role for CA in the tumour was suggested by the observation that the tumour microenvironment is more acidic than normal tissues [172]. As has been discussed in section 1.6. this was initially attributed to tumour cell related metabolism. More recent work has suggested that the reversal of the normal intracellular-extracellular pH gradient is in part due to overexpression of tumour cell membrane bound CA. This abnormal pH homeostasis favours tumour progression by enhancing tumour invasion, suppressing the host immune system and may inhibit the uptake of some cytotoxic drugs.

The discovery of an inducible membrane bound CA molecule, CA IX, identified an enzyme that could be responsible for the maintenance of this abnormal pH gradient. Pastorekova et al. initially discovered CA IX in 1992 when it was labelled with the nomenclature MN. The protein was found have a central CA domain. CA IX was found to be inducible either by keeping the cells in dense cultures or infecting them with a transmissible agent MX (coding for p58X protein) [411, 412]. CA IX appeared to be a tumour associated protein as it was expressed by a series of transformed cells but not by their normal counterparts. In agreement with this contention fibroblasts transfected with CA IX displayed typical characteristics of transformed cells such as loss of contact inhibition, a change in morphology, shorter doubling time, loss of anchorage dependence and decreased dependence on growth factors. Immunohistochemistry studies have reported frequent membranous tumour cell expression [413]. Electron microscopy visualised CA IX on the surface microvilli and in the nucleus, particularly in the nucleoli [413].

In light of these findings, a series of studies questioned whether CA IX expression could be used as a biomarker of neoplastic change. Expression of CA IX has been shown to be useful marker for neoplastic lesions in papanicolaou smears, bronchial and renal biopsies that were difficult to detect using conventional methods [109, 110, 414]. Conversely, CA IX is expressed under physiological conditions by the gastric, intestinal and gallbladder epithelium, but is suppressed in gastric malignancies [415]. In DCIS and invasive cancer of the breast and SCCHN, increased expression is associated with markers of an aggressive phenotype and/or a poor prognosis [106, 178, 180].

A second membrane bound member of the CA family has been discovered in association with cancer, CA XII [416]. Unlike CA IX, CA XII is more commonly expressed in normal tissue and less frequently upregulated in DCIS and clear cell renal carcinoma [178, 414, 416]. In DCIS, CA XII expression was found to be associated with markers of a good prognosis such as an absence of necrosis and low grade in contrast to CA IX [178].

4.1.4. CA IX and XII are under oxygen dependent -regulation

Identification of endogenous markers of hypoxia will aid investigators study tumour cell behaviour in a hypoxic microenvironment. They may also be used to identify patients with highly hypoxic tumours, enabling tailoring of therapeutic strategies. Both CA IX and XII are induced by hypoxia. The transcription of both may be regulated by HIF-1α as tumour cell lines that have mutated VHL protein, overexpress CA IX and XII [179]. This has been confirmed for CA IX as a HRE has been identified in its promoter region [179, 417]. There is mounting evidence that CA IX can be used as an endogenous hypoxic marker in different solid tumours. Expression of CA IX is seen predominantly around areas of tumour necrosis. It has a similar staining pattern to pimonidazole, which is a recognised exogenous marker of hypoxia. A study found a large degree of overlap between pimonidazole and CA IX staining in sequential tumour sections [179, 418, 419]. Finally a prospective study found a close correlation between polaro-graphic measurements of intratumoural oxygen tensions and CA IX immunostaining in cervical carcinoma [124].

Table 4.1. Cellular locations of carbonic isoenzymes

Isoenzyme	Anatomical location and role	Reference
CA I (c)	Erythrocyte, vascular smooth muscle, GI tract, eye (less active than CA II), gastric mucosa, adrenal gland	[420-424]
CA II (c)	Kidney, salivary gland, gall baldder & pancreas, eye, gastric mucosa, adrenal gland, brain	[421-427]
CA III (c)	Erthrocytes, muscle, eye (less active than CA II)	[422, 428]
CA IV (m)	Kidney, GI tract male reproductive system, CNS, alveoli	[423, 425, 427, 429-432]
CA V (mit)	Liver & kidney (generates HCO_3^- for pyruvate carboxylase), pancreatic β cells (insulin secretion)	[433, 434]
CA VI (s)	Kidney, salivary gland & pancreas, milk (development of infant alimentary tract), pancreas	[425, 435]
CA VII (c)	Non-enzymic, salivary gland	[436]
CA VIII	CNS	[437]
CA IX (m)	Gastric, intestinal and gall bladder epithelia	[415]
CA X (c)	Non-enzymic, CNS	[437]
CA XI	Non-enzymic, CNS	[437]
CA XII (m)	Kidney, pancreas, colon	[427, 438]
CA XIV	Kidney, liver	[439]

4.1.5. Aims of this chapter

(a) To examine the frequency of different patterns of CA IX staining in NSCLC.

(b) To further assess the use of CA IX as a marker of tumour cell hypoxia by:

(i) Estimating the pO_2 at which tumour cells start to express CA IX.

(ii) Comparing the distances from blood vessels to a CA IX positive tumour cells and to CA IX negative tumour cells.

(c) To evaluate the association between CA IX expression and clinicopathological and the biological variables previously studied in this patient series.

(d) To assess the prognostic significance of CA IX expression in surgically resected NSCLC.

4.2. METHODS

From the series of 181 suitable patients 4 were excluded due to lack of available tissue, for description of the population see section 2.3. The Envision kit was used to visualise immunopositivity of M75 antibody against CA IX, see section 2.2.3.5.5. The IHC method is summarised in table 2.1. The interpretation of CA IX staining is described in section 2.7.2. The random selection of cells and measurement of the distance from the selected cells to the nearest microvessel is described in section 2.8. The estimation of the pO_2 at which membranous CA IX staining starts is described in section 2.9. Statistical analysis is described in section 2.12. The antibodies used for the immunolocalisation of EGFR, MMP-2, MMP-9, bcl-2 and p53 staining by G. Cox are outlined in section 3.3

4.3. RESULTS

4.3.1. CA IX tumour cell expression

Tumour cell CA IX immunostaining was detected in 80.2% (142) of patients. Mixed patterns of cytoplasmic (c) and membranous (m) expression were seen in the majority of patients (figure 4.1a) (table 4.2). The median % for both mCA IX and cCAIX staining patterns was 5% (range 0-100%). mCA IX and cCA IX expression was observed in lung carcinoma *in-situ* and entrapped bronchial epithelium within the tumour mass.

Figure 4.1.a. Perinecrotic NSCLC mCA IX and cCAIX staining

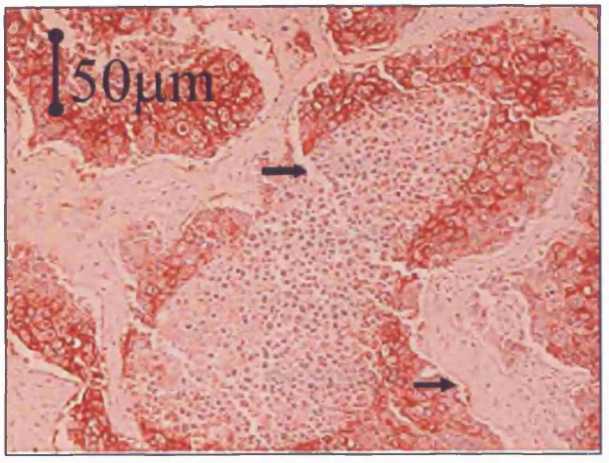


Figure 4.1b. Circumferential pCA IX staining

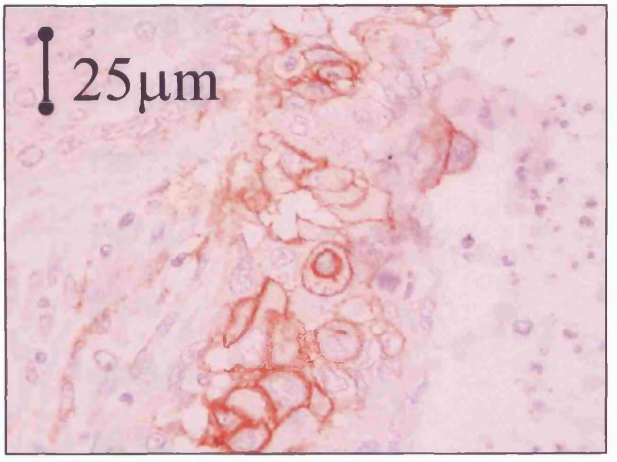
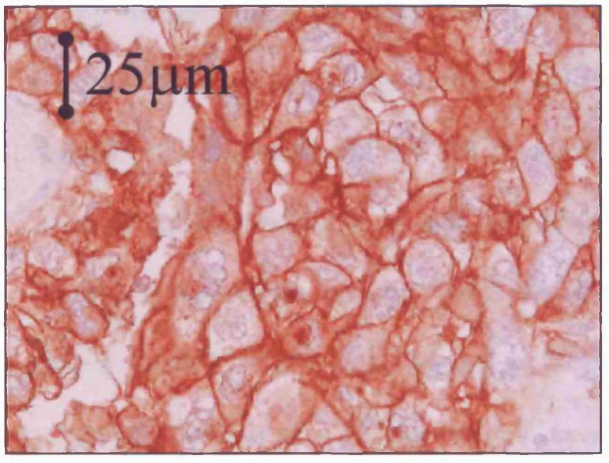


Figure 4.1c. pCA IX staining indenting the nucleus



pCA IX staining obscuring the nucleus

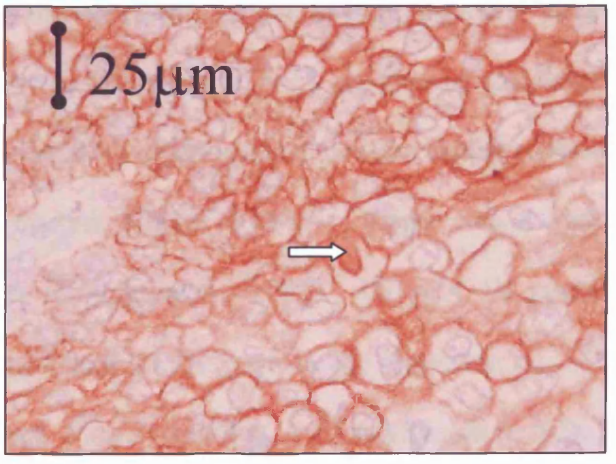


Table 4.2. CA IX Tumour Cell Expression

Pattern of Tumour Cell Expression	No. of Patients (%)
Membranous	136 (76.8)
Cytoplasmic	129(72.8)
Membranous and cytoplasmic	123(77)
Membranous only	13(7.3)
Cytoplasmic only	6(3.4)
Perinuclear	46(25.9)
Nuclear	3(1.7)
No expression	33(18.6)

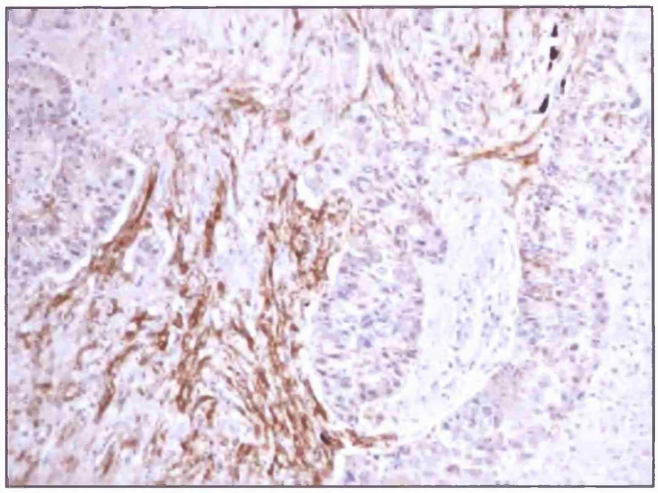
Perinuclear (p)CA IX expression was observed in occasional discrete tumour cells that always had mCA IX expression. There were 3 distinct patterns classed as pCA IX expression: (a) staining obscuring the nuclear structures, (b) circumferential staining of the nuclear margins and (c) discrete areas of staining adjacent to and indenting the nucleus (figure 4.1b-d.). There was a highly significant association between mCA IX and pCA IX staining ($p<0.001$). Of pCA IX positive patients (n=46), 95.6% had high mCA IX expression and 66.7% were in the extensive mCA IX subgroup. Positive pCA IX expression also correlated with increased cCA IX expression ($p<0.001$). Three patients with pCA IX expression also expressed nuclear CA IX.

4.3.2. CA IX Stromal Cell Expression

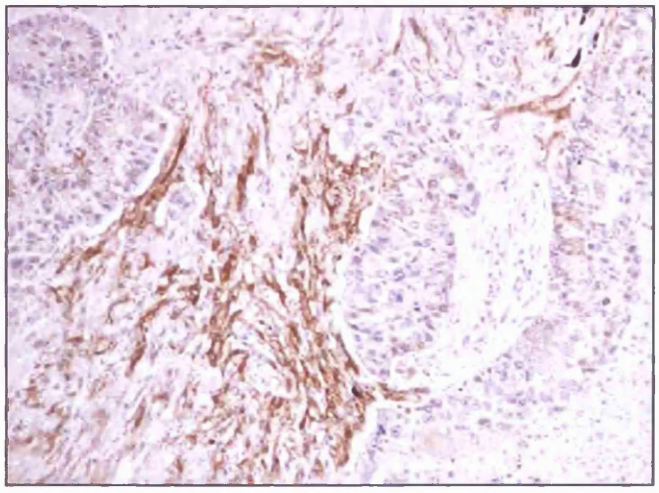
Stromal cell (s)CA IX expression was observed in 111 (62.7%) patients. Serial sections were stained with CD-31 and CD-34, markers for vascular endothelium, and vimentin and smooth muscle

actin (SMA), markers for myofibroblasts [197, 235, 440]. A clear overlap was seen between CA IX and SMA indicating the sCA IX positive cells were activated myofibroblasts (figure 4.2.).

Figure 4.2. Comparison of M75 staining and SMA staining in adjacent tumour sections



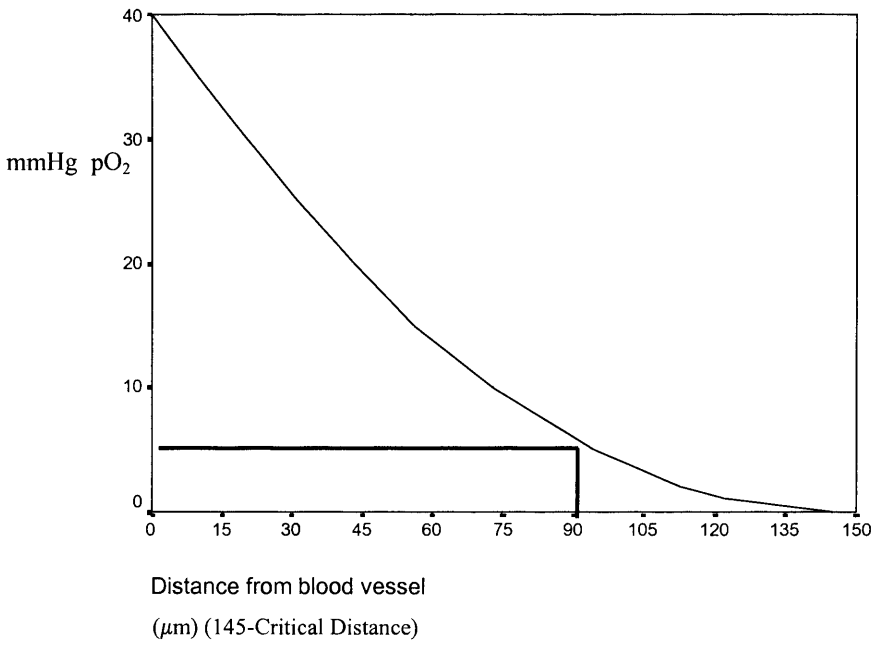
Activated fibroblast marker: Smooth muscle actin staining



Stromal CA IX staining

4.3.3. Distance from vascular endothelium to the start of CA IX tumour positivity

The median distance from the vascular endothelium to the tumour cells expressing CA IX was 90μm (range 49-143μm, n=18). The measured distances were an approximation as it was not possible to quantify tissue shrinkage during section to slide fixation. Thomlinson and Gray published a formula, which calculates the critical distance from a blood vessel to where TN commences. The calculations were based on examination of histological specimens of squamous cell lung carcinoma. The formula makes assumptions of oxygen diffusion and oxygen consumption. Accepting that necrosis occurs when the $pO_2 = 0$ the formulae can be used to estimate the pO_2 a set distance from a blood vessel. The pO_2 90μm from the blood vessel is calculated to be 5.77mmHg or 0.76% O_2 (figure 4.3.) [353].



$$\text{Critical Distance (cm)} = \sqrt{\frac{4 \times O_2 \text{ diffusion coefficient (DC)} \times pO_2(\text{mmHg})}{O_2 \text{ consumed/ ml/ second (M)}}}$$

$DC/M = 1.31 \times 10^{-6}$

Figure 4.3. A graphic representation of Thomlinson and Gray's equation estimation the pO_2 a set distance from a blood vessel

4.3.4. Mean tumour cell to blood vessel distances for randomly selected cells

There was a significant difference between the tumour to blood vessel distances for mCA IX positive tumour cells (mean =117μm, STD 54μm, n=26) and mCA IX negative tumour cells (mean distance=43μm, STD=34μm, n=61) ($p<0.001$). There was also a significant difference between the tumour to blood vessel distances for tumour cells from areas with less than the median percentage of tumour cells expressing mCA IX (mean=38μm, STD=22μm, n=47) than from areas with greater than the median percentage of tumour cells expressing mCA IX (mean=104μm, STD=54μm, n=40) ($p<0.001$), irrespective of whether or not the actual cell selected was mCA IX positive.

4.3.5. CA IX associations with clinico-pathological and biological variables

High mCA IX expression was associated with squamous histology ($p=0.006$), TN ($p=0.004$), epidermal growth factor receptor (EGFR) expression ($p=0.009$), pCA IX ($p<0.001$), and a near significant association was found with p53 ($p=0.08$) (table 4.3.).

Positive pCA IX expression was associated with EGFR expression ($p<0.001$), squamous histology ($p=0.003$) and a near significant association was found with platelet count ($p=0.093$) (table 4.4.). pCA IX expression was not associated with TN ($p=0.12$).

Increased sCA IX expression was associated with high mCA IX ($p=0.003$) but not with either pCA IX or cCA IX. sCA IX was significantly associated with advanced tumour stage ($p=0.011$) and TN ($p<0.001$).

Table 4.3. mCA IX associations with clinicopathological and biological variables

Prognostic Factor		< Median mCA IX	≥ Median mCA IX	χ^2 p value
EGFR	< Median	53	31	0.009
N=176	≥ Median	40	52	
MMP-2	Negative	61	51	0.23
N=168	Positive	25	31	
MMP-9	Low	44	35	0.16
N=166	High	39	48	
pCA IX	Negative	90	41	<0.001
N=177	Positive	3	43	
P53	< 20%	56	44	0.08
N=177	≥ 20%	37	40	
Bcl-2	< 20%	59	56	0.65
N=177	≥ 20%	34	28	
MVD	< Median	46	40	0.94
N=173	≥ Median	46	41	
TN	Limited	63	39	0.004
N=177	Extensive	30	45	
Stage	I	50	35	0.17
N=177	II	21	29	
	IIIA	22	20	
T-Stage	1	18	13	0.33
N=177	2	67	58	
	¾	8	13	
N-Stage	0	52	40	0.41
N=177	1	24	30	
	2	17	14	
Gender	Female	27	25	0.92
N=177	Male	66	59	
Histology	Not Squamous	44	23	0.006
N=177	Squamous	49	61	
Platelet count	< Median	43	43	0.59
N=171	≥ Median	46	39	

Table 4.4. pCA IX associations with clinicopathological and biological

Prognostic Factor		Negative pCA IX	Positive pCA IX	χ^2 p value
EGFR	< Median	73	11	<0.001
N=176	≥ Median	58	34	
MMP-2	Negative	82	30	0.95
N=168	Positive	41	15	
MMP-9	Low	61	18	0.18
N=166	High	59	28	
P53	< 20%	77	22	0.18
N=177	≥ 20%	54	24	
Bcl-2	< 20%	84	31	0.69
N=177	≥ 20%	47	15	
MVC	< Median	63	23	0.69
N=173	≥ Median	66	21	
TN	Limited	80	22	0.12
N=177	Extensive	51	24	
Stage	I	63	22	0.65
N=177	II	35	15	
	IIIA	33	9	
T-Stage	1	23	8	0.71
N=177	2	94	31	
	3/4	14	7	
N-Stage	0	67	25	0.91
N=177	1	42	13	
	2	22	8	
Gender	Female	39	13	0.85
N=177	Male	92	33	
Histology	Not Squamous	58	9	0.003
N=177	Squamous	73	37	
Platelet count	< Median	58	28	0.093
N=171	≥ Median	67	18	

4.3.6. Subgroup analysis of mCA IX associations with TN and EGFR

mCA IX expression was divided into quartiles (negative, low, moderate and extensive expression) as opposed to binary groups to further examine the association with TN. The linear by linear association test but not the chi square test found that increasing TN was associated with increasing expression of mCA IX (table 4.5.). The loss of significance, when using the chi squared test, was due to their being no significant differences between the extent of TN in the extensive group compared to the no and low expression groups. For example in 53% (n=25) of patients in the extensive subgroup there was limited TN. In 31% (n=12) of the no expression subgroup there was extensive TN. In contrast when mCA IX subgroups were compared to EGFR expression the association was retained (p=0.012) and the linear by linear association was stronger (p=0.002) (table 4.5).

Table 4.5. Frequency tables of mCA IX staining divided 4 groups and TN and EGFR

4 mCA IX subgroups	Focal TN	Extensive TN	χ^2 test	Linear by linear association
Negative	27	12		
Low	27	13		
Moderate	23	28		
Extensive	25	22	0.054	0.036
	EGFR < 20%	EGFR ≥20%		
Negative	26	14		
Low	21	17		
Moderate	21	30		
Extensive	16	31	0.012	0.002

4.3.7. Univariate and multivariate survival analysis

Using univariate analysis pCA IX expression (p=0.036) was an indicator of a poor prognosis (figure 4.4a). mCA IX (p=0.11), cCA IX (p=0.27) and sCA IX (p=0.95) expression were not associated with prognosis (table 4.6). Co-expression of EGFR with pCA IX was more strongly associated with a poor prognosis than pCA IX alone (p=0.003) (figure 4.4b). Using multivariate analysis this change was shown to be significant (table 4.7.).

In the full multivariate analysis pCA IX was included with the clinico-pathological and biological variables discussed in chapter 3. MVD (p=0.001), tumour stage (p=0.005), platelet count (p=0.007), pCA IX expression (p=0.003) and gender (p=0.017) were independent poor prognostic factors (table 4.8).

Table 4.6. Univariate analysis for different patterns of CA IX staining.

Prognostic Factor		n	Hazard Ratio	95% CI	p value
CA IX Perinuclear	Positive	46	1.0		0.044
	Negative	131	1.50	1.03–2.19	
CA IX Membranous	< median	93	1.0		0.11
	≥ median	84	1.32	0.94–1.85	
CA IX Cytoplasmic	< median	103	1.0		0.27
	≥ median	74	1.21	0.86–1.71	
CA IX Stromal	0 – 2	109	1.0		0.95
	2 – 3	68	0.99	0.70-1.40	

Figure 4.4a. pCA IX Kaplan Meier survival curves

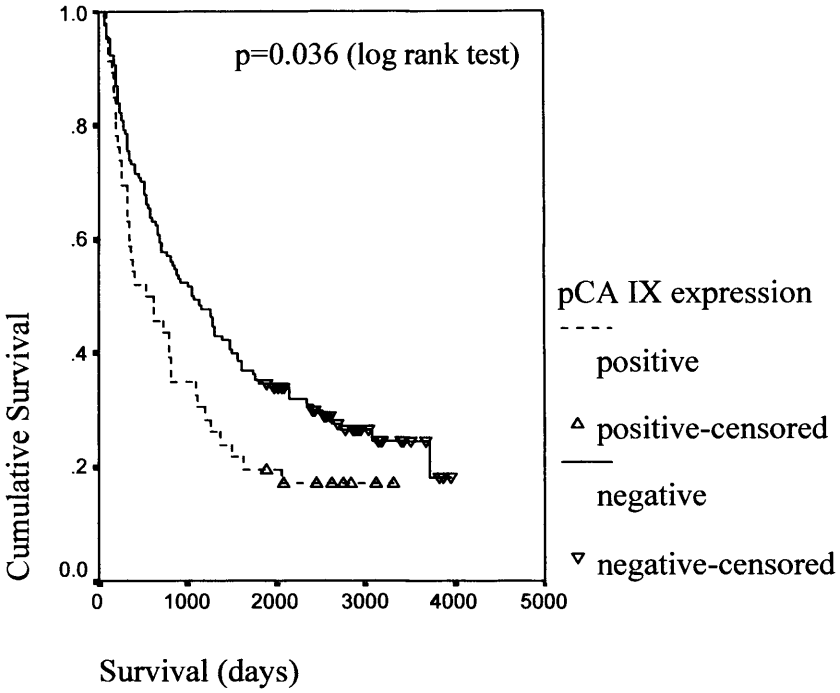


Figure 4.4b. pCA IX/ EGFR coexpression Kaplan Meier survival curves

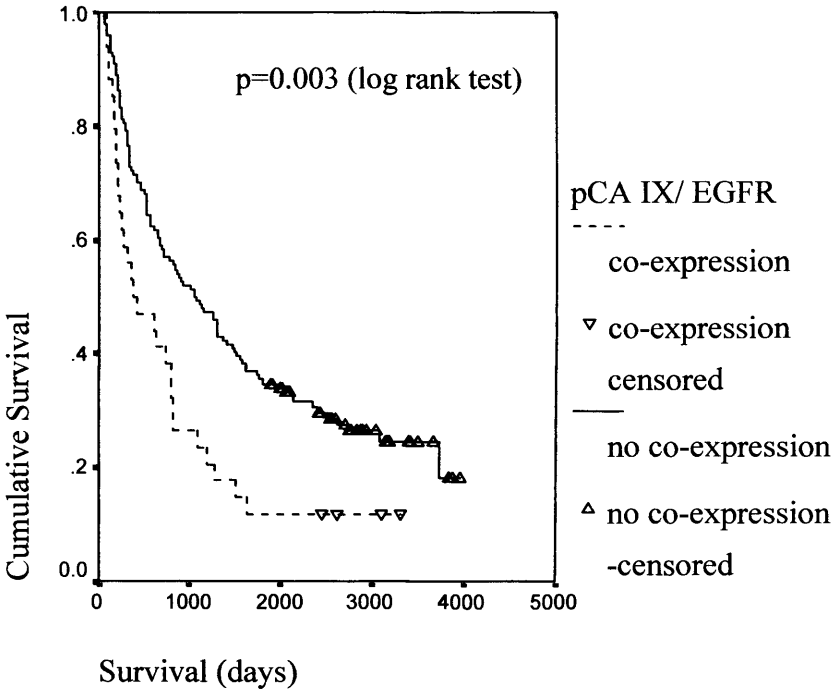


Table 4.7. Multivariate analysis of EGFR, pCA IX and EGFR / pCA IX co-expression

Variable		Harzard Ration	95% CI	p value
pCA IX	Negative			0.92
	Positive			
EGFR	< 20%			0.33
	≥ 20%			
pCA IX/ EGFR	Negative	1.0		0.003
	Positive	1.87	1.24-2.82	

Table 4.8. Multivariate analysis for pCA IX staining and clinico-pathological and other biological variables

Factor		Hazard Ratio	95% CI	p-value
TMN Stage	I	1.00		0.005
	II	1.61	1.02 – 2.50	0.037
	IIIA	2.06	1.3-3.28	0.002
Gender	Female	1.00		
	Male	1.71	1.1-2.65	0.017
Platelet Count	< median	1.00		
	≥ median	1.71	1.16-2.52	0.007
MMP-9	< 20%	1.00		
	≥ 20%	1.63	1.11-2.39	0.013
MVD	< median	1.00		
	≥ median	1.89	1.29-2.77	0.001
pCA IX	Negative	1.00		
	Positive	1.91	1.25-2.91	0.003

4.4. DISCUSSION

This study has demonstrated that different patterns of CA IX expression are widely expressed in NSCLC and that expression varies greatly between patients. The frequency (81.8 %) and predominant focal perinecrotic pattern of CA IX expression in this cohort is similar to that found in a previous series of 65 NSCLC patients (80%) [109]. The perinecrotic nature of the membranous and cytoplasmic pattern of expression is consistent with findings from studies in invasive breast cancer, DCIS of the breast, cervical cancer and SCCHN [106, 124, 178, 180].

The association of TN with mCA IX, and sCA IX expression is in keeping with the proposal that CA IX is upregulated by and may be used as a marker of tumour hypoxia [106, 124, 178, 180]. The estimation of the pO_2 (5.77mmHg or 0.76% O_2) at which CA IX expression becomes apparent is similar to Beasley *et al.* findings who used the same method in SCCHN tissue sections. *In vitro* studies have also reported that CA IX is increasingly upregulated as the pO_2 is decreased from 2.5% to 0.1% [106, 179].

The deduction that CA IX is a marker of hypoxia based on the strength of the estimated pO_2 may be flawed. The technique used incorporates bias by only selecting tumour sections that have extensive expression and by excluding blood vessels that have been cut along their longitudinal axis. In order to counter this bias a second technique was used. We randomly selected individual cells and measured the distance to the nearest blood vessel. Two comparisons were made. Firstly, distances measured from mCA IX positive cells were compared to distances measured from mCA IX negative cells. Secondarily, distances were measured from cells within areas of high mCA IX expression and were compared with distances measured from cells within areas of low mCA IX expression, irrespective of whether the selected tumour cell was CA IX positive or negative. In both cases a highly significant difference was found with mCA IX positivity being associated with an increased distance from blood vessels. The mean distance from blood vessels to tumour cell positivity was

similar for both the first technique and the distances measured using the second technique. The second technique found a somewhat longer mean distances (117μm and 104μm –v- 90μm). This discrepancy would be expected as the former method only looked at cells that were in a close proximity to the reference blood vessel where as the second technique selected a random cell and measured the distance to the nearest blood vessel. These findings add new evidence to support the aforementioned postulate that CA IX is a marker of tumour cell hypoxia. Also the relative lack of CA IX staining immediately adjacent to microvessels suggests that these tissues are well oxygenated, and as by inference, these microvessels are active.

The fact that a range of distances was found from positive CA IX tumour cells to blood vessels implies that either CA IX was being upregulated at different O_2 saturations or the O_2 saturation at a set distance from a blood vessel varies throughout the tumour. The latter explanation is the more likely for several reasons. O_2 utilization may vary throughout the tumour due to differences in the rate of tumour cell proliferation. Evidence for this is provided by many immunohistochemical studies that show a great heterogeneity of expression of both growth and apoptotic factors by individual cells within the tumour mass [64, 384]. Secondly, blood flow through the tumour microvessels may be intermittent causing variations in the degree of hypoxia at any set distance from the microvessel. This is due to the highly irregular tumour microvasculature, where vessels are frequently immature, vessel compression common and blood flow unreliable (see section 1.3.)

pCA IX expression was associated with a poor prognosis and in multivariate analysis was independent of stage, MMP-9 expression, platelet count, gender and MVD. Addition of pCA IX to the multivariate analysis removed TN from the equation. The lack of association between mCA IX expression and prognosis was surprising as increased tumour hypoxia promotes a more aggressive tumour phenotype and mCA IX expression has been shown to be prognostic in other solid tumours [96, 106, 124, 180]. Cytoplasmic CA IX was also not associated with prognosis. pCA IX expression

may represent a subset of tumours with either an enhanced response to hypoxia or a highly hypoxic microenvironment, either of which could be responsible for producing a relatively more aggressive cancer. Evidence supporting this statement comes from the observation that pCA IX expression was only seen in mCA IX positive cells and sections with pCA IX had predominantly extensive mCA IX expression. The lack of an association with TN would suggest that pCA IX cases are not highly hypoxic or if they are, are able to escape necrotic cell death (see later). In addition to the lack of association between pCA IX expression and TN, subgroup analysis of mCA IX expression found little difference between the degree of TN present in the moderate and extensive mCA IX subgroups to explain the difference in CA IX staining. These findings suggest a secondary mechanism is involved in the regulation of CA IX. HIF-1 regulates CA IX transcription; hence aberrant increased expression of HIF-1 would be expected to result in increased expression of CA IX.

Disregulation of HIF-1 can be caused by mutation of the VHL tumour suppressor gene with subsequent loss of O_2 dependent regulation. Overexpression of CA IX is a common finding in tumours with this mutation [121, 179]. VHL gene mutations have previously been demonstrated in a proportion of cell lines derived from thoracic malignancies [441]. These mutations may be present in a subgroup of patients causing pCA IX and extensive mCA IX expression in the absence of a hypoxic stimulus.

A second stimulus that may cause increased CA IX expression in the absence of high levels of hypoxia could be activation of the PI-3K pathway. Increased activity of the PI-3K pathway when stimulated by EGF has been shown to increase HIF-1α expression independently of O_2 in tumour cell lines [278]. Furthermore activated EGFR is able to enhance the hypoxic induction of the HIF-1α transcriptional target, VEGF via the PI-3K pathway [280]. Similarly EGFR activation may also induce CA IX in concert with hypoxia in NSCLC. The relationships between both TN and EGFR expression with pCA IX and extensive mCA IX expression support this contention. Alternatively

EGFR signalling may indirectly induce CA IX expression by stimulating increased tumour cell proliferation or inhibiting apoptosis thereby increasing O_2 consumption and worsening hypoxia.

The importance of the relationship between EGFR and CA IX is highlighted by the survival analysis showing EGFR/ pCA IX co-expression confers a significantly worse prognosis than pCA IX alone.

The structures responsible for pCA IX positivity are unknown. This pattern of staining may be CA IX protein synthesis in the endoplasmic reticulum. The finding that pCA IX was predominantly found in cases with extensive mCA IX expression where an increased rate of synthesis would be expected, supports this. Similar perinuclear patterns of staining for the adrenocorticotrophin receptor have been demonstrated to be protein retained in the endoplasmic reticulum by counterstaining with endoplasmic reticulum markers [442].

TN was commonly observed in the absence of CA IX staining. This may represent infarction of non-hypoxic tissue caused by an acute ischaemic event, as has been described in chapter 3.

sCA IX expression was correlated with nodal and overall stage but not prognosis. The relationship between stage and sCA IX expression suggests the stroma of tumours with increased metastatic potential is more hypoxic. The morphological appearances of the stromal cell type expressing CA IX were initially thought to be either clusters of fibroblasts or microvessels. Staining of serial sections with cell type markers indicated that the CA IX positive stromal cells were activated myofibroblasts. Previously tumour fibroblasts have been shown to express other hypoxia inducible factors such as VEGF [443].

A higher frequency of mCA IX and pCA IX expression was observed in squamous carcinomas than other histological categories, which is possibly due to squamous carcinomas being more necrotic than other NSCLC histological subtypes [444]. Alternatively EGFR is more frequently expressed in this histological subtype and may be responsible for this finding [64].

Entrapped non-malignant bronchial epithelium and, in particular lung carcinoma *in situ,* often expressed mCA IX. This reflects the hypoxic nature of these tissues, in contrast to adjacent normal lung tissue that never stained for CA IX. These observations are in keeping with previous studies in NSCLC and cervical cancer where CA IX expression has been proposed to be a marker of neoplastic change [109, 110].

CA IX may be a therapeutic target for cancer. Inhibition of CA isoenzymes with acetozolamide or sulphonamides results in either reduced tumour invasiveness or blocked tumour growth, respectively [337, 445]. Furthermore CA isoenzyme antagonism has been observed to augment the cytotoxic effects of various chemotherapeutic agents, including platinum based drugs [446]. Further work is required to investigate whether these results are reproducible *in vivo*. Work is under way investigating the possibilities of using CA IX as a target for antigen specific immunotherapy in renal carcinoma where it is widely expressed in the tumour but not in normal tissue [345].

In summary pCA IX expression has been identified as a prognostic variable in NSCLC. The associations with TN and the spatial relationships between tumour CA IX expression and blood vessels indicate that CA IX expression can be used as a surrogate marker of hypoxia in NSCLC. A small subset of tumours had high mCA IX expression in the presence of low or absent TN. This discrepancy and the associations with EGFR indicate regulation of this enzyme by factors other than hypoxia in a proportion of patients. More work is required to identify the structures expressing pCA IX and to elucidate all the regulatory processes involved in the regulation of the enzyme.

Chapter 5

Hypoxia Inducible Factors-1α and -2α in NSCLC

5.1. INTRODUCTION

5.1.1. Hypoxia inducible factors

A number of different transcription factors have been identified that are upregulated by and mediate the cellular response to hypoxia [447]. HIF-1 was identified by Semenza and colleagues in 1992 [448]. HIF-1 is a heterodimer that consists of HIF-1α and HIF-1β, otherwise known as the aryl hydrocarbon receptor nuclear translocator (ARNT). These subunits belong to the basic helix-loop-helix (bHLH)-PAS protein family and are constitutively expressed in the cytoplasm of eukaryotic cells. HIF-2α is an isoform of HIF-1α and also interacts with HIF-1β [144, 145]. HIF-2α has previously been referred to as the endothelial PAS domain protein 1, HIF-1α like factor, member of PAS superfamily 2 and mouse HIF-related factor [144, 145, 449, 450].

5.1.2. Oxygen dependent regulation of HIF-1α and 2α (HIF-α) subunits

The half-life of HIF-α subunits is very short under normal O_2 saturations (normoxia) but increases exponentially with decreasing O_2 saturations. HIF-1β expression is not influenced by changes in O_2 saturations [451, 452]. The O_2 saturation regulates HIF-α subunit proteolysis [453-456]. The initial step of proteolysis of both HIF-α subunits is ubiquitination followed by uptake and degradation by the 26S proteosome [454, 456]. The ubiquitination step involves a critical interaction between the tumour suppressor protein pVHL, a component of the multiprotein ubiquitin E3 ligase complex and HIF-α subunits [121, 457]. The β-domain of pVHL acts as a bridging protein between the E3 ligase complex and the HIF-α subunits [122]. Mutation of the pVHL gene results increased expression of both HIF-α subunits [146].

An oxygen-dependent degradation domain (ODDD) has been identified in the centre of the HIF-1α molecule. Within the HIF-1α ODDD two independent regions, spanning residues 556-572 and

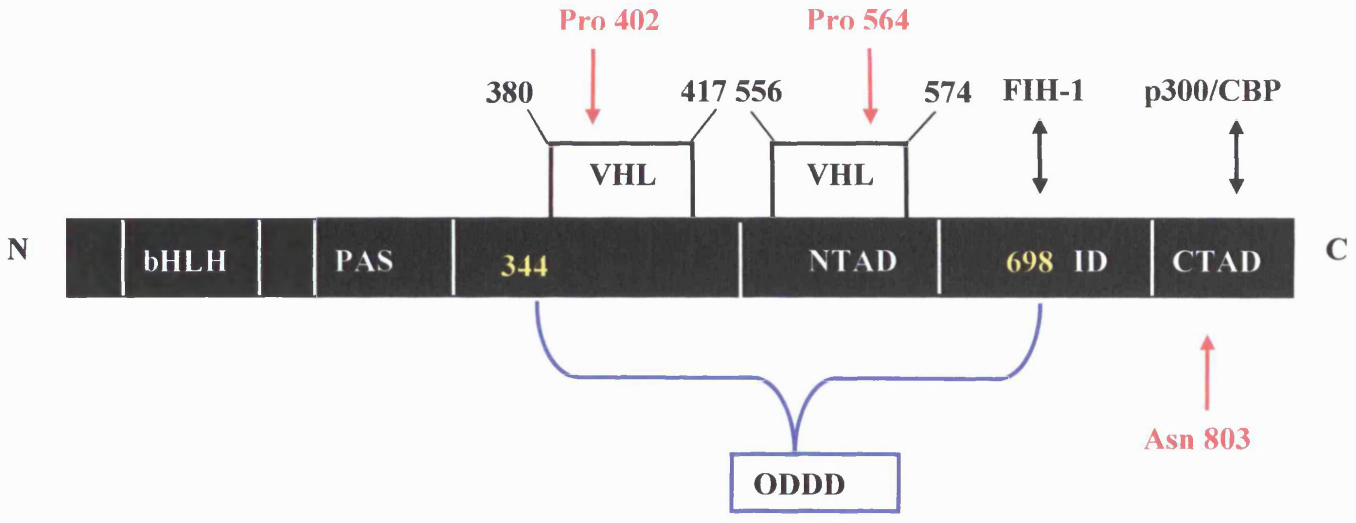
380-417 interact with the pVHL (figure 5.1) [454, 458, 459]. This interaction depends on the hydroxylation status of prolyl residues 564 and 402 within these 2 regions [458, 460, 461]. The prolyl hydroxylase enzymes responsible for this hydroxylation are dioxygenases. These enzymes utilize O_2 as a co-substrate and ferrous iron as a co-factor, so providing a direct link between the availability of O_2 and the regulation of HIF-1α. This mechanism explains why the iron chelator, desferrioxamine induces HIF-1α expression.

For full transactivational activity the C-terminal activation domain (C-TAD) of HIF-1α interacts with the transcriptional co-factors CREB binding protein (CBP)/p300 and SRC-1 (figure 5.1) [462-465]. Hydroxylation of an asparagine residue in the C-TAD inhibits the interaction between these transcriptional co-factors and HIF-1α. Factor-inhibitory HIF (FIH) has been identified as the enzyme responsible for asparagine hydroxylation, which is also a dioxygenase hydroxylase and hence inhibited by hypoxia. Therefore hypoxia influences both the level of expression and transcriptional activity of HIF-1α [466, 467].

Regulation of HIF-2α expression and transcriptional activity conforms to a similar model through hydroxylation of prolyl residue 531 in the ODDD and asparagine residues in the C-TAD [468, 469].

Although the regulation of HIF-1α and HIF-2α appear similar there are differences in their pattern of expression. In mouse embryo and human tumours HIF-2α expression is less restricted to hypoxic regions than HIF-1α expression, suggesting that there are as yet unresolved differences in the oxygen-dependent regulation of these 2 factors [144, 145, 149].

Figure 5.1. The structure of HIF-1α



5.1.3. Oxygen independent regulation of HIF-α

HIF-1α is also regulated via oxygen independent mechanisms. Oxygen dependent degradation of HIF-1α is inhibited by the v-Src and Ras V12 oncogenes [468]. Growth factors, cytokines and oncogenes stimulating the MAPK pathways and/ or the PI-3K pathways have been reported to increase HIF-1α activity. The importance of these signaling pathways to the regulation of HIF-1α differs in amongst cell lines from different tumour types and may be cell type specific [292, 470], [293] (see section 1.9.).

5.1.4. Functions of HIFs

HIF-1 and HIF-2 stimulate transcription of genes that contain a HRE. These target genes are involved in a wide range of processes that promote cellular survival. Such processes include angiogenesis, blood vessel vasodilation and erythropoesis, anaerobic metabolism, buffering of the intracellular compartment and induction of growth factors (see sections 1.6.-1.8.).

HIF-1α activity *in vivo* has been found to promote tumour growth in the majority of studies and resistance to various chemotherapy agents including platiniums [97, 127, 128, 471]. Contrary to

these survival mechanisms, HIF-1α may also promote apoptosis and tissue necrosis (see section 1.5.). Indeed a study by Carmeliet *et al.* reported a tumour suppressor effect *in vivo* [129]. The phosphorylation status of HIF-1α may determine whether it acts to protect or check tumour cell survival. Dephosphorylated HIF-1α stabilises p53 thereby inducing apoptosis whereas phosphorylated HIF-1α binds to HIF-1β to form the HIF-1 complex [131].

5.1.4. Hypoxia inducible factor-α expression in solid tumours

HIF-1α and HIF-2α are widely expressed in solid tumours. HIF-1α has been found to be associated with both an improved and a worse prognosis in different tumour types, the results of which are summarized in table 5.1. In NSCLC, a recent study found no significant association between HIF-1α and prognosis but did not specify the cut point used [141]. Similarly, studies report different results for the prognostic implications of HIF-2α in solid tumours. In NSCLC the same paper that reported the findings for HIF-1α found that HIF-2α expression was associated with a poor prognosis. However the cell type evaluated was not specified [141]. In tumour associated macrophage's HIF-2α expression has been investigated in breast cancer and SCCHN. An association with a poor prognosis was found in the former but not the latter [141, 142, 151].

Table 5.1. Summary of IHC of HIF-1α studies in solid tumour

Tumour type	Stage	Treatment	Primary antibody	Pattern of expression	Cut point (%)	number	P value survival	Ref
Cervical	Early	Surgery	Mab H1α67 NB 100-105	Nuclear	> 4 points	91	<0.0001‡	[143]
Cervical	Locally advanced	R/T	Mab H1α67 NB 100-105	Nuclear	2%	45	n/s	[140]
NSCLC	I-IIIA	Surgery	ESEE 122	Nuclear/ cytoplasmic	?	108	0.08‡	[141]
Oropharyngeal	All	R/T +/- chemo	Mab H1α67 NB 100-105	Nuclear	Tertiles 10% +50%	98	0.001‡	[139]
Oesophageal	Early	PDT+/- R/T	ESEE 122	Nuclear/ cytoplasmic	?	37	0.04‡*	[138]
SCCHN	Early	Surgery	ESEE 122	Nuclear	Positive or negative	79	0.027†	[142]
SCCHN	Locally advanced	Chemo R/T	ESEE 122	Nuclear/ cytoplasmic	Mean value not quantified	75	0.05‡	[137]
Oligodendroma		Surgery+/- chemo R/T	Mab H1α67 NB 100-105	Nuclear	>4 points	51	0.043‡	[136]
Breast	Lymph node positive	Surgery+/- chemo+/- tamoxifen	Mab H1α67 NB 100-105	Nuclear	quartiles	206	0.001‡	[135]
Epithelial Ovarian	I-IV	Surgery adjuvant chemo	Mab H1α67 NB 100-105	Nuclear	quartiles	102	n/s	[134]

Abbreviations in table: R/ T: radiotherapy; PDT: photodynamic therapy.

*Response to photodynamic therapy not survival;†good prognosis;‡poor prognosis; n/s: non-significant

5.1.5. Aims of chapter 5

(a) To optimise immunohistochemical methods for staining for HIF-1α and HIF-2α.

(b) Describe the cell types and pattern of staining of HIF-1α and HIF-2α.

(c) Compare the patterns and extent of expression of HIF-1α and HIF-2α with clinico-pathological and biological variables.

(d) Correlate the extent of expression of HIF-1α and HIF-2α with prognosis.

5.2. METHODS

5.2.1. Optimisation of HIF-1α and 2α immunihisyochemisty

5.2.1.1. HIF-1α

During optimization, the positive controls used initially in method 1 were sections of clear cell renal carcinoma that were known to express HIF-1α. The primary antibody was anti HIF-1α ESEE 122 monoclonal antibody that was donated by Prof. A. Harris. Subsequently, a NSCLC case that was found to have strong HIF-1α expression was used. For negative controls the primary antibody was omitted. The method tried are summarised in table 5.2. and are discussed below.

5.2.1.1.1. Method 1.

The first method used had previously been used in a descriptive paper charting the expression of HIF-1α in different tumour and tissue types [149]. The antigen retrieval process involved prolonged incubation at low temperature (60°C) in a water bath and the Envision kit to expose immunopositivity, discussed in sections 2.2.3.2.2 and 2.2.3.5.5.

Results: 'Negative control' was negative. Positive control: A strongly positive 'positive-control' was identified that consistently expressed both nuclear and membranous expression (figure 5.2a).

Faint nuclear staining was the most common staining pattern observed (figure 5.2b). As staining was weak a second positive control was included that had less intense staining than the initial positive control. This was used to assess reproducibility of weaker staining. Staining was scored as 0 as no staining, <10%=1, 10%-<30%=2 and ≥30%=3. A high level of intra-run variability was found that was unacceptable (table 5.3.).

Table 5.2. Methods used in optimisation of HIF-1α

Method	Antigen Retrieval	Antibody Clone	IHC kit	Staining problem
1	W/b 16hrs 60°C EDTA pH 8	Anti HIF-1α ESEE 122	Envision	Very faint, inconsistent
	W/b 16hrs 60°C EDTA pH 8	Anti HIF-2α ESEE190b	Envision	Very faint
2	M/w 2x 4mins in citric acid pH 6.0	Anti HIF-1α ESEE 122	APAAP	Background staining, inconsistent and patchy
3	P/c 2 or 4mins in citric acid pH 6.0	Anti HIF-1α ESEE 122	APAAP	Background staining
4	P/c 3 mins in citric acid pH 6.0	Anti HIF-1α ESEE 122	Envision	Background staining
5	P/c 3 mins in citric acid pH 6.0	Anti HIF-1α ESEE 122	ABC	Faint non-specific
6	W/b 40 mins Dako AR solution	H1α67	CSA	Short $t_{1/2}$ of CSA kit
7	P/c 3 mins EDTA pH 8.0	Anti HIF-2α ESEE 190b	2-step	Inconsistent

Abbreviations used in table: w/b: water bath; m/w: microwave; p/c: pressure cooking.

Figure 5.2. Anti-HIF-1α ESEE 122 staining in NSCLC

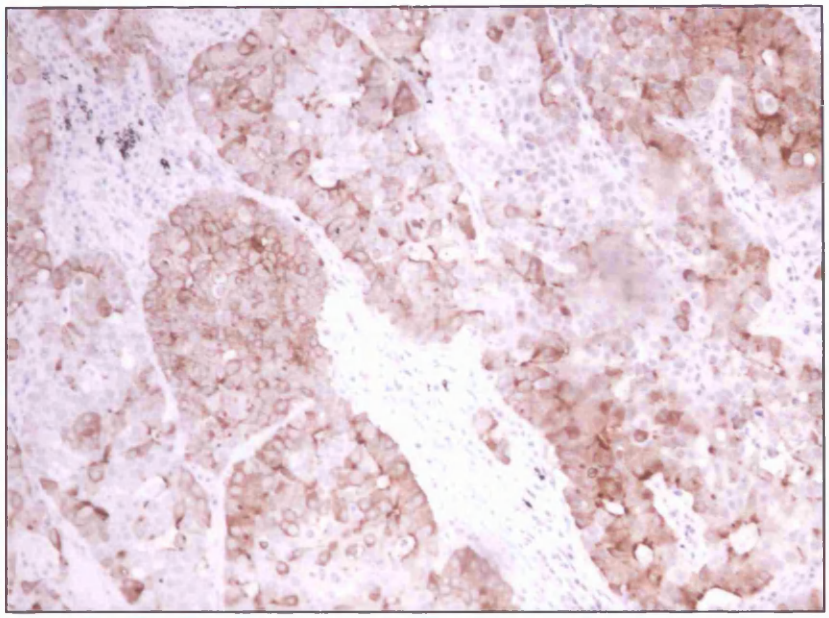


Figure 5.2a. Positive control demonstrating membranous, cytoplasmic and nuclear staining

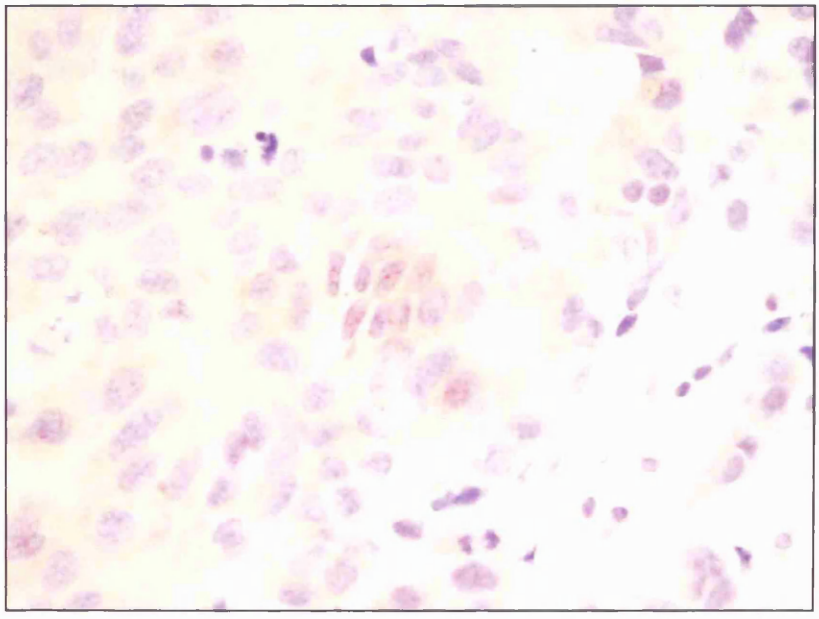


Figure 5.2b. Predominant staining pattern: faint nuclear staining

Table 5.3. Inconsistency of results from method 1

	HIF-1α score	
Run number	Control slide 1	Control slide 2
1	3	1
2	3	0
3	3	3
4	3	1
5	3	3
6	3	2
7	3	0
8	3	3

5.2.1.1.2. Method 2

Using the same antibody a second method has been published that had investigated the prognostic implications of HIF-1α overexpression in NSCLC [141]. This method used a more aggressive antigen retrieval process, pressure-cooking for 3 minutes and detected immunopositivity using the alkaline phosphatase anti-alkaline phosphatase (APAAP). These techniques are described in sections 2.2.3.2.1 and 2.2.3.5.

Results: 'Negative control' was negative. Positive control: Wide spread cytoplamsic staining was observed that was difficult to distinguish from background staining. The extent of staining was patchy and inconsistent with intra-run and inter-run variability observed (table 5.4.). Faint or moderate nuclear staining was observed in 2/10 cases.

Table 5.4. Variability in the results using HIF-1α immunohistochemistry method 2

Case number	Run 1	Run 2	Run 3
1	70% cyto	5-10% cyto	50% cyto
2	60% cyto 5%nuc	5-10%cyto <5% nuc	
3		15% cyto *	15% cyto *
4		50% cyto	30% cyto
5		30%cyto 10% nuclear	40% cyto 10% nuclear

*inconsistent staining as the positivity was in non-overlapping parts of the tumour sections

5.2.1.1.3. Methods 3-5.

Method 3. To attempt combat the patchy and inconsistent staining the antigen retrieval process was changed to pressure cooking for 2 or 4 minutes and the fuscin exposure shortened from 25 to 15 minutes.

Results: 'Negative control' was negative. Positive control: The background staining persisted and the APAAP technique was abandoned.

Method 4. Slides were pressure cooked for 3 minutes in citric acid and the immunopositivity detected by the Envision kit. Results: 'Negative control' was negative. Positive control: excessive background staining again made interpretation impossible.

Method 5. Slides were pressure cooked for 3 minutes in citric acid and the immunopositivity detected using the ABC technique, described in section 2.2.3.5.4. Results. 'Negative control' was negative. Positive control: very faint non-specific staining that was not interpretable.

5.2.1.1.4. Method 6.

After 5 methods had failed to provide clear and reproducible staining the primary antibody was changed. Zhong *et al.* published a study in locally advanced cervical cancer using the anti-HIF-1α monoclonal antibody clone Mab H1α67, NB 100-105 (Novus Biologicals, Liddleton, CO, USA) [472]. The techniques for antigen retrieval and staining are described in sections 2.2.3.2.3 and 2.2.3.5.6. (method 6, table 2.1.).

Results: 'Negative control' was negative. Positive control: Uniform and reproducible staining with minimal background was achieved. The only problem encountered was that the CSA kit seemed to have a relatively short shelf life. This manifested as an increase in background staining with successive runs. The background staining promptly disappeared when a new kit was purchased. The kits were stored in accordance with manufacturers guidelines at 4°C. The staining was completed with multiple CSA kits, the only problem being a pecuniary one. Although this problem has not been mentioned in published texts a MD student working with the same antibody independently of our group has encountered the same problems.

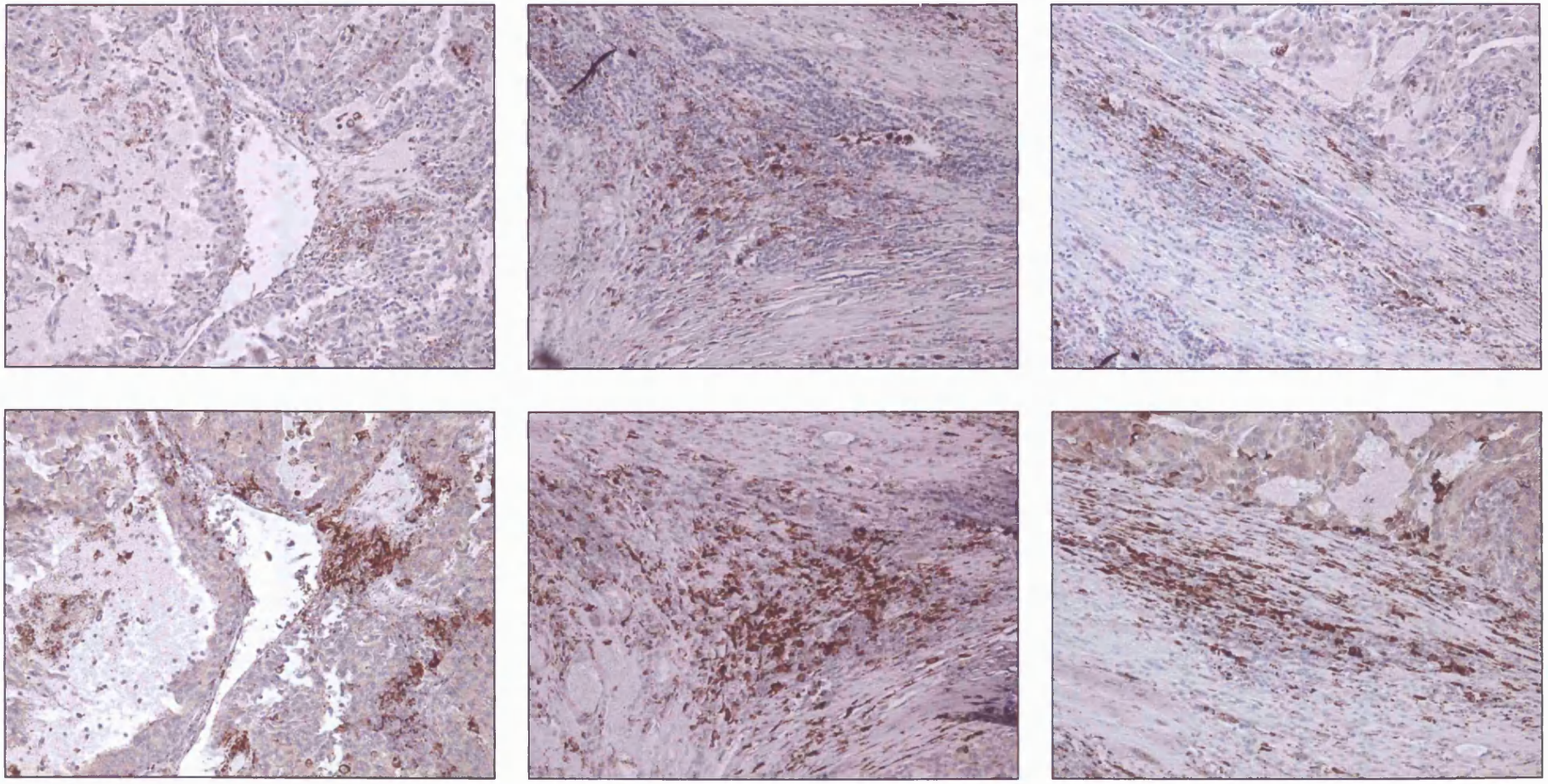
5.2.1.2. HIF-2α

The descriptive paper that outlined HIF-1α staining used method 1 used the same method to stain for HIF-2α using Anti-HIF-2α EP190b. Again this technique was attempted but similar problems were encountered as with HIF-1α. Very faint staining was difficult to interpret. The poor staining was grounds enough to abandon this technique and try a different published method.

Method 7. Using the same antibody Leak et al. published a study in breast cancer using pressure-cooking in EDTA pH 8.0 and the 2-step indirect immunohistochemical technique to visualise immunopositivity. This is described in section 2.2.3.5.2 (method 7, table 2.1.) [151].

Results. Relatively clean staining was achieved with little background staining (figure 5.3.). The positive controls were consistently positive and checked by H. Turley in Oxford who has experience using this antibody and is a co-author of several related papers [141, 142, 149, 151, 473]. The initial method of interpretation is described in section 2.7.4. Although the positive controls were consistently positive the extent of staining seemed to vary in repeated NSCLC sections (figure 5.3.). As a result of this the initial scoring system was abandoned and the sections were scored as either positive or negative.

Figure 5.3. Variability of anti-HIF-2α ESEE190b in Non-small cell lung cancer



Control slide 1

Control slide 2

Control slide 3

5.3. Summary of study population and methods

From the series 9 patients were excluded due to lack of available tissue. For description of the population studied see section 2.3. For description of the interpretation of HIF-1α and HIF-2α see sections 2.7.3. and 2.7.4. For description of the statistical methods used see section 2.12. The antibodies used for the immunolocalisation of EGFR, MMP-2, MMP-9, bcl-2 and p53 staining are outlined in section 3.3 and the cut points used to define the extent of staining as either high or low are tabulated in table 3.2.

5.4. RESULTS

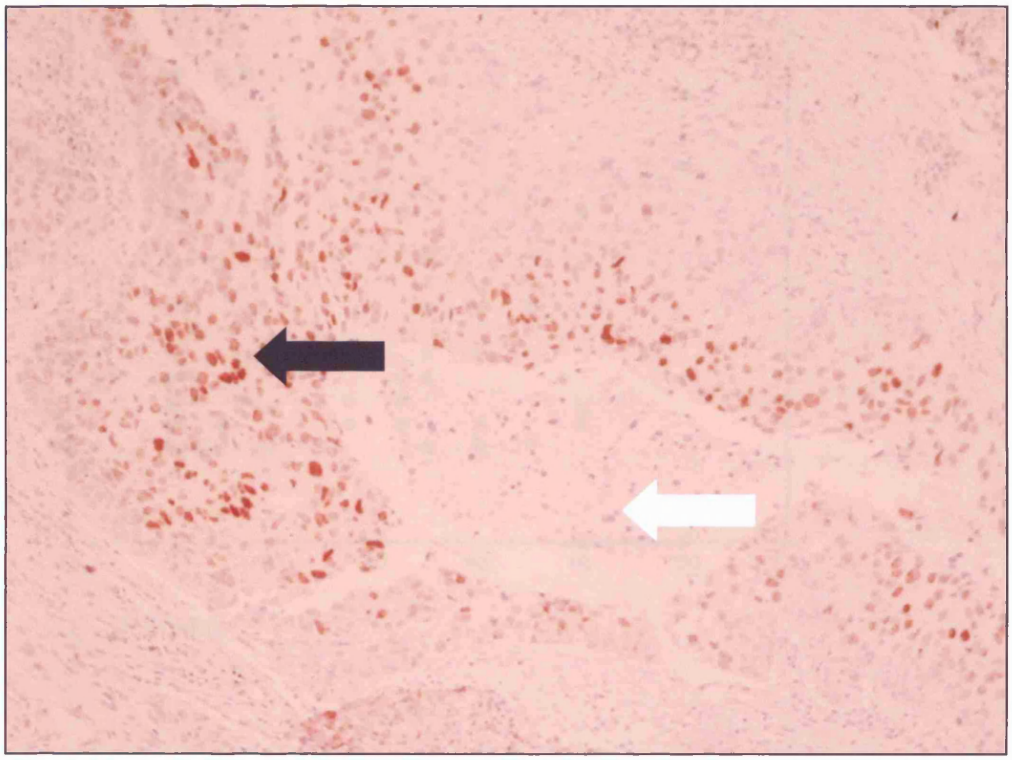
5.4.1. Tumour expression of HIF-1α and HIF-2α

5.4.1.1. HIF-1α

Using method 1, 127 sections were stained and interpreted for HIF-1α. Nuclear HIF-1α expression was observed in 95 (74%) patients and cytoplasmic expression was observed in 8 (7.2%) patients. Of these 8 patients, 6 had staining in 1%, 1 patient had staining in 10% and 1 had staining in 20% of cells. Cytoplasmic expression could not be assessed in 16 patients due to background staining. The extent of HIF-1α nuclear expression varied considerably, range 0-80%.

Using method 6, 172 cases were available for staining for HIF-1α. Nuclear HIF-1α expression was observed in 97 cases (60.4%). A number of runs displayed convincing cytoplasmic staining. However this was shown to be background staining as it occurred in the negative controls unlike the nuclear staining that did not. The median percentage of positive tumour cells was 5%, range 0-100%. The staining was predominantly perinecrotic (figure 5.4.). No expression was observed in either tumour stroma or adjacent normal tissue.

Figure 5.4. Anti-HIF-1α H1α67 staining in Non-small cell lung cancer

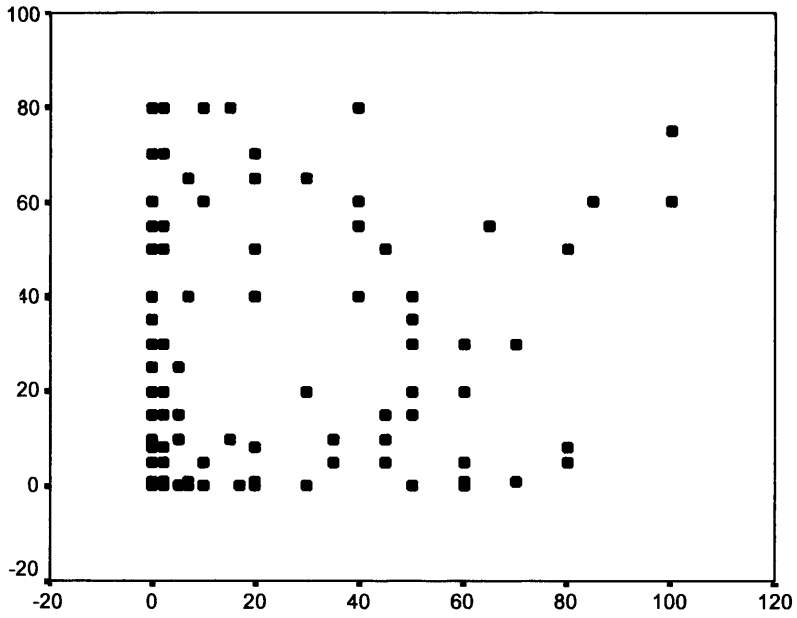


Black arrow: peri-necrotic tumour cell nuclear HIF-1α staining

White arrow: Tumour necrosis

There was no association found between the extent of nuclear expression detected using methods 1 and 6 (figure 5.5.).

Figure 5.5. Scatter plot of nuclear staining of HIF-1α assessed by methods 1 and 6



a Method 6.

5.4.1.2. HIF-2α

Using method 7, 117 cases were stained for HIF-2α. Nuclear staining was observed predominantly in macrophages. Staining was rarely observed within the tumour mass. Expression of HIF-2α was less restricted to perinecrotic areas than HIF-1α (figure 5.6).

Positive HIF-2α expression was observed in 73 patients (64.6%). Again like HIF-1α the extent of positivity varied greatly between patients, using the initial scoring system the median score was 0.63, range 0–5. No association was found between positive HIF-2α immunoreactivity and HIF-1α expression (p=0.95).

Figure 5.6. Anti-HIF-2α ESEE 190b staining in NSCLC

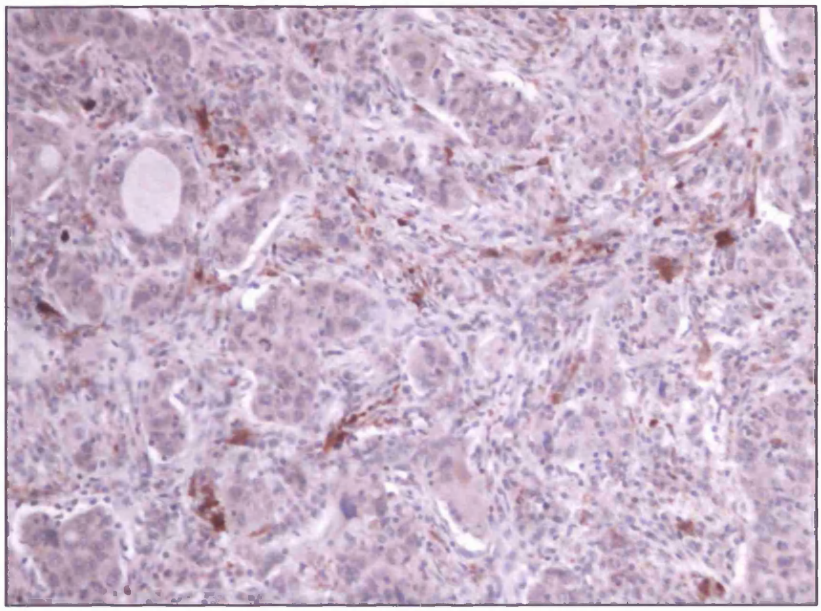


Figure 5.6a. Stromal macrophages adjacent to squamous tumour cells expressing HIF-2α

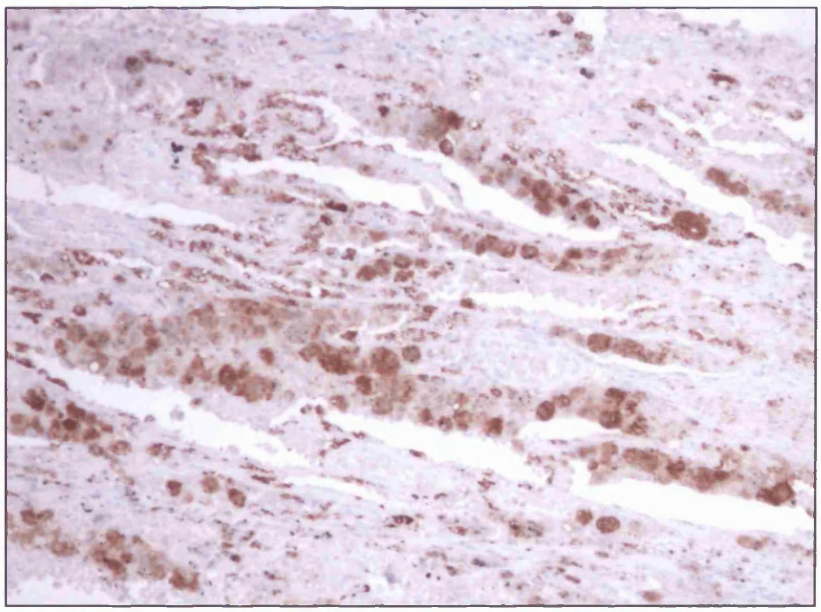


Figure 5.6b. Macrophages in abnormal non-malignant tissue at edge of tumour mass expressing HIF-2α

5.4.2. Associations with clinico-pathological and biological variables

5.4.2.1. HIF-1α

No further statistical analysis was performed using the data generated by method 1 due to the lack of consistent staining. From data generated using method 6 positive HIF-1α expression (defined as equal to or above the median) was associated with the following clinico-pathological variables: increasing T-stage ($p=0.042$), squamous histology ($p<0.001$) and cases with extensive TN ($p<0.001$). No association was found with N- or overall stage, gender or MVD.

Positive HIF-1α expression was also positively associated with the following biological factors: EGFR ($p=0.01$), pCA IX ($p=0.004$) and mCA IX ($p<0.001$), MMP-9 ($p<0.003$) and p53 ($p=0.008$). A non-significant trend towards an association was found with MMP-2 ($p=0.07$). No association was found with Bcl-2 ($p=0.25$) (table 5.5).

High HIF-1α expression (defined as equal to or above 60%) was associated with mCA IX ($p=0.001$), pCA IX ($p=0.006$), TN ($p=0.003$) and squamous histology ($p=0.001$).

Table 5.5. HIF-1α associations with clinico-pathological and biological variables

Prognostic Factor		Negative HIF-1α	Positive HIF-1α	χ^2 p value
Stage	I	48	37	0.63
	II	24	26	
	IIIA	20	17	
T-Stage	1	18	13	0.042
	2	69	53	
	3/4	5	14	
N-Stage	0	51	41	0.48
	1	24	28	
	2	17	11	
Histology	Not Squamous	52	13	< 0.001
	Squamous	40	67	
Platelet count	< Median	43	43	0.59
	≥ Median	46	39	
TN	Limited	69	32	< 0.001
	Extensive	23	48	
MVD	< Median	45	41	0.58
	≥ Median	47	36	
EGFR	< 20%	53	30	0.01
	≥ 20%	39	49	
MMP-2	Negative	52	55	0.07
	Positive	35	20	
MMP-9	< 20%	51	26	0.003
	≥ 20%	36	48	
mCA IX	< Median	60	28	< 0.001
	≥ Median	30	51	
pCA IX	Negative	73	49	0.004
	Positive	16	30	
Bcl-2	< 20%	64	49	0.25
	≥ 20%	28	31	
P53	< 20%	60	36	0.008
	≥ 20%	32	44	

5.4.2.2. HIF-2α associations with clinico-pathological and biological variables

No assocation was found between positive HIF-2α expression and any of the clinico-pathological or biological variables studied. Specifically, unlike HIF-1α, no association was found with TN.

5.4.3. Survival analysis for HIF-1α and HIF-2α

There was a near significant association with prognosis for HIF-1α expression when the percentage of staining was used as a continuous variable (p=0.07). When the series was dichotomised into categorical variables high but not positive HIF-1α expression was prognostic (p=0.035 and 0.52, respectively) (figures 5.6 and 5.7).

Positive HIF-2α expression was not associated with prognosis.

High HIF-1α (p=0.006) was an independent prognostic variable when the clinico-pathological variables stage, gender and platelet count were analysed in multivariate analysis (table 5.6). When novel clinico-pathological variables MVD and TN and biological variables pCA IX and MMP-9 were included in multivariate analysis high HIF-1α was not an independent variable. Stage (p=0.023), MVD (p=0.001), gender (p=0.012), platelet count (p=0.007), pCA IX (p=0.002) and MMP-9 (p=0.027) were independent variables (table 5.7).

Figure 5.7. Kaplan Meier survival curve for HIF-1α using the median as the cut point

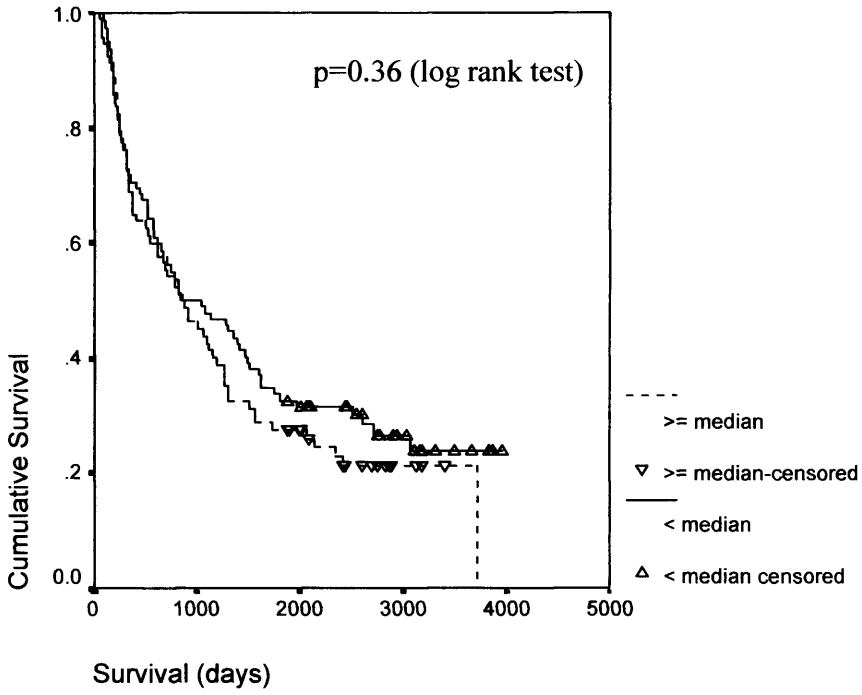


Figure 5.8. Kaplan Meier survival curve for HIF-1α using the ≥ 60% as the cut point

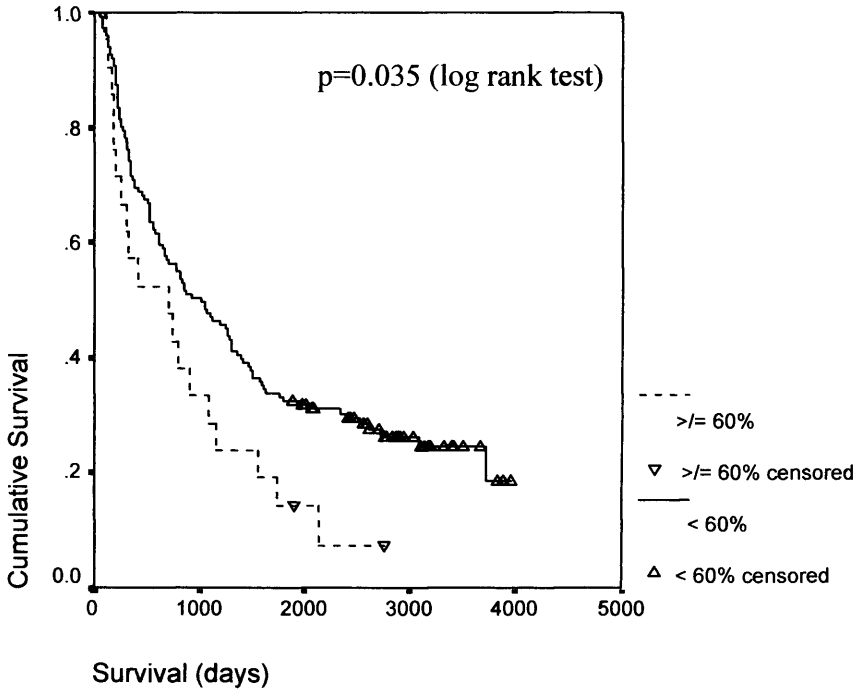


Table 5.6. Multivariate analysis for HIF-1α and routine clinico-pathological variables

Variable		Hazard ratio	95% confidence intervals	p value
Stage	I	1		<0.001
	II	1.74	1.15-2.64	
	IIIA	2.29	1.47-3.53	
HIF-1α	< 60%	1		0.006
	≥ 60%	2.05	1.23-2.44	
Gender	Female	1		0.027
	Male	1.60	1.06-2.44	

Table 5.7. Multivariate analysis for all clinico-pathological and biological variables

Variable		Hazard ratio	95% confidence intervals	p value
MVD	< Median	1		0.001
	≥ Median	1.92	1.3-2.83	
pCA IX	Negative	1		0.002
	Positive	1.96	1.28-3.01	
Platelets	Low	1		0.007
	High	1.72	1.16-2.56	
Gender	Female	1		0.012
	Male	1.77	1.13-2.78	
Stage	I	1		0.023
	II	1.56	1-2.44	
	IIIA	1.84	1.14-2.99	
MMP-9	< 20%	1		0.027
	≥ 20%	1.56	1.05-2.3	

5.6. DISCUSSION

This study demonstrates that HIF-1α and HIF-2α are commonly expressed in NSCLC. Using both monoclonal anti-HIF-1α antibodies, nuclear and cytoplasmic expression was observed. For the second anti-HIF-1α antibody, Mab anti-H1α67 the cytoplasmic expression was shown to be background as it was also present on the negative controls. These results are consistent with previous studies that report only nuclear staining using anti-HIF-1α Mab H1α67 but report both nuclear and cytoplasmic expression when using anti-HIF-1α Mab ESEE 122 [141, 143]. The 2 antibodies bind to different epitopes. The lack of cytoplasmic expression with Mab H1α67 suggests that the epitope it binds to may be masked when the protein presides in the cytoplasm. Unfortunately no consistent staining was achieved using the anti-HIF-1α ESEE 122 antibody. Furthermore the interpretation of the staining that was achieved was difficult, as the positivity was very faint. Using techniques to increase the intensity of the positivity resulted in increased background staining and as a result the primary antibody was changed. Using the anti-HIF-1α Mab H1α67 clear and reproducible staining was obtained that was mainly adjacent to areas of necrosis. The fact that the results obtained using the 2 antibodies did not correlate casts further doubt over the reliability of the first antibody's staining.

HIF-2α expression was observed pre-dominantly in macrophages and although positivity was occasionally observed in tumour groups this may have been the result of infiltrating macrophages. This pattern of staining was also reported in breast cancer where a similar technique was used [151]. The previous study in NSCLC reported both tumour cell and macrophage positivity [141]. The differences may be a result of the different techniques used to detect immunopositivity. Unlike HIF-1α, HIF-2α expression appeared to be less localised to areas of necrosis, which is in agreement with previous studies in solid tumours [149]. Although HIF-2α positive controls were consistently

positive there was a degree of inter-run variability in the extent of staining. In view of this variability it is difficult to draw definite conclusions from the lack of associations with other variables and prognosis. Work in breast cancer has implicated macrophages as a source of HIF-2α regulated angiogenic growth factors and correlated increased macrophage counts with angiogenesis and prognosis [151, 152, 378, 474]. The finding of widespread expression of HIF-2α in macrophages in NSCLC suggests a similar paradigm might occur in this tumour type.

Using the second Mab anti-HIF-1α, H1α67, HIF-1α nuclear expression was associated with a number of biological factors that are involved in the pathogenesis of NSCLC and high HIF-1α expression was associated with a poor prognosis. In multivariate analysis high HIF-1α was independent of routine clinico-pathological variables, gender and stage but was not independent of novel clinico-pathological and biological variables.

The prognostic significance of HIF-1α expression has now been evaluated in a number of solid tumours (table 5.1.). Increased HIF-1α expression has been reported to be associated with a poor prognosis in lymph node positive breast cancer, non-metastatic oropharangeal cancer and oligodendroma [135, 136, 139]. Conflicting results have been reported in cervical carcinoma and in SCCHN. In early stage cervical carcinoma treated with surgery HIF-1α expression was found to be strongly associated with a poor prognosis. In contrast in locally advanced cervical carcinoma treated with radiotherapy HIF-1α was not prognostic. This was surprising as HIF-1α expression in the latter study did correlate with tumour hypoxia, an established poor prognostic factor in this disease [140, 143]. In the study in early stage SCCHN treated with surgery, the presence of HIF-1α expression conferred a good prognosis as opposed to the study of locally advanced disease, treated with chemo-radiotherapy, where an association with a poor prognosis was found [137, 142].

A possible explanation for the variability in the results seen may be due to the cut points used to define cases as over expressing HIF-1α. In the studies that did not show HIF-1α to be associated with a poor prognosis, the cut-points used were relatively low, being ≤ 5% of HIF-1α positive cells. In contrast, studies reporting HIF-1α to be associated with a poor prognosis have used higher cut points. An exception is a study of epithelial ovarian carcinomas where the series was divided into quartiles and no association was found with prognosis. In fact HIF-1α expression correlated with the apoptotic rate especially in low malignant potential tumours (borderline tumours). However in cases with strong HIF-1α expression *i.e.* the upper quartile and p53 co-expression an association with a poor prognosis was observed [134]. Taking the evidence from these separate studies into consideration, the results suggest that high HIF-1α expression may need to be present in order for the transcription factor to have a pro-tumourigenic effect. This postulate is also in agreement with studies demonstrating that HIF-1α has both pro- and anti-tumour growth properties (see section 5.1.4.) and is in keeping with our findings.

The fact that CA IX, the extent of TN and squamous histology were all associated with HIF-1α is consistent with the recognised mechanisms that regulate and are regulated by HIF-1α. As has been discussed in the previous chapter discussion CA IX is upregulated by HIF-1α and squamous carcinomas are known to be more necrotic than other NSCLC histological subtypes.

The associations of HIF-1α with EGFR and MMP-9 have not previously been reported in immunohistochemical studies. As was discussed in the previous chapter EGFR may directly or indirectly upregulate HIF-1α and thereby induce CA IX expression. The finding that HIF-1α expression is also closely associated with EGFR expression adds weight to this postulate.

MMP-9 is an endopeptidase that facilitates tumour invasion and angiogenesis through breakdown of the extracellular matrix. MMP-9 mRNA levels are regulated by a number of transcription factors

including nuclear factor-kappa B and activator protein-1, which can be induced by the p42/p44 MAPK and PI-3K pathways in response to cytokines and growth factors [475, 476]. To date HIF-1α has not been implicated in the regulation of MMP-9 although some *in vitro* work has found the enzyme is induced by hypoxia [395, 396]. This induction of MMP-9 could be due to increased NF-Kappa B transcriptional activity that can also be induced by hypoxia [477]. The correlation between HIF-1α and MMP-9 raises the possibility that HIF-1α may have a regulatory role in the expression of this protease or it may reflect the fact that both factors may be induced by similar stimuli such as hypoxia or EGF [64, 292]. More work is required to elucidate whether or not HIF-1α is directly involved in the regulation of MMP-9. HIF-1α was not associated with MMP-2 expression. In keeping with this, and in contrast to MMP-9, hypoxia has not been shown to induce MMP-2 expression in tumour cell lines [396].

The association between HIF-1α and p53 suggests that either HIF-1α stabilizes wild type p53 or mutant p53 induces HIF-1α in NSCLC [131, 478]. The fact that there was no association between prognosis and co-expression of HIF-1α and p53 is consistent with activity of both these mechanisms (data not shown) [384]. *In vitro* studies have shown that Bcl-2 and HIF-1α may regulate the activity of each other [129, 479, 480]. No association was found between Bcl-2 and HIF-1α making it unlikely that either consistently influences the expression of the other in NSCLC.

Previous studies have reported conflicting evidence as to whether HIF-1α expression is associated with MVD [134, 136, 137, 141, 142]. No association was found between HIF-1α expression and MVD in our patient series. The regulatory processes governing angiogenesis are complex and the predominant stimulus may vary in different parts of the tumour. For example hypoxia has been shown to be the dominant stimulus governing VEGF expression in perinecrotic areas of tumours but in the periphery of tumours transforming growth factor (TGF)-β1 has been

shown to be more important [481]. In our series the MVD was exclusively evaluated at the tumour edge, which may therefore mask any association between HIF-1α and MVD [197].

In summary we demonstrate that HIF-1α is widely expressed in NSCLC as opposed to adjacent normal tissue. The associations with EGFR, p53 and CA IX support data from other studies that these factors are either involved in the regulation of, or are in part regulated by HIF-1α. The association with MMP-9 suggests that this enzyme is also a transcriptional target of HIF-1α. The association between high HIF-1α expression and a poor prognosis suggests that an increasingly hypoxic environment plays a role in potentiating the pro-tumorigenic properties of this transcription factor.

Chapter 6

Can EGF Induce Carbonic Anhydrase IX?

6.1. INTRODUCTION

The epidermal growth factor (EGF) promotes tumour cell proliferation, inhibits apoptosis and induces angiogenesis by activating its cognate receptor EGFR. EGFR is a member of the c-erb B transmembrane bound tyrosine kinase receptor family, first identified in 1980 [482]. As discussed previously in section 1.11. EGFR is a target for therapy in NSCLC using selective tyrosine kinase inhibitors and monoclonal antibodies [65-67]. Activation of EGFR results in increased activity of a number of intracellular pathways. Of these the PI-3K and p42/ p44 MAPK pathways have been shown to influence the activity of HIF-1α in tumour cell lines (see section 1.9.). As previously described in chapter 4 CA IX is regulated by HIF-1α. In chapters 4 and 5 we found that strong interrelationships existed between the expression of EGFR, HIF-1α and CA IX that supported the contention that EGFR signaling may induce CA IX either independently of or additively with hypoxia.

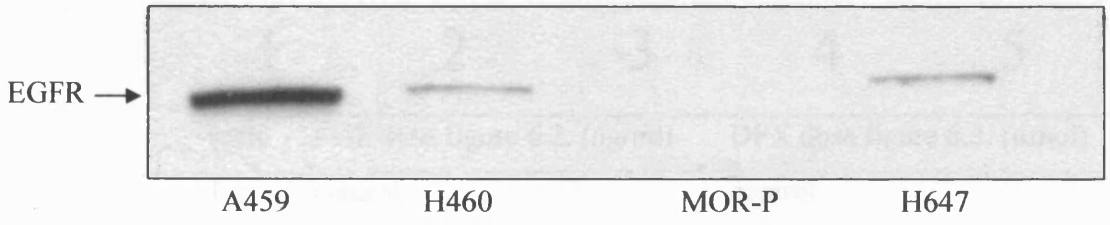
In this chapter we investigated this potential phenomenon by culturing an EGFR positive NSCLC cell line and evaluating CA IX expression after exposing this to:

1. EGF
2. Desferrioxamine (DFX), a hypoxia mimetic
3. ZD1839, an EGFR tyrosine kinase inhibitor
4. Differing cell density

6.2. METHODS

Tissue culture and western blot methods are outlined in section 2.10 and 2.11. In summary A549 cells were seeded and grown to confluence or sub-confluence and serum starved for 24-48 hours during which time any pre-treatments were administered. Cells were then treated with appropriate factors and harvested at set time points. The expression of CA IX was analysed in cell lysates by Western blotting using the M75 monoclonal antibody. A549 lung adenocarcinoma cells were used as they expressed a high level of EGFR (figure 6.1).

Figure 6.1. NSCLC blot demonstrating EGFR expression in different cell lines. A549 was selected for subsequent experiments.



6.3. RESULTS

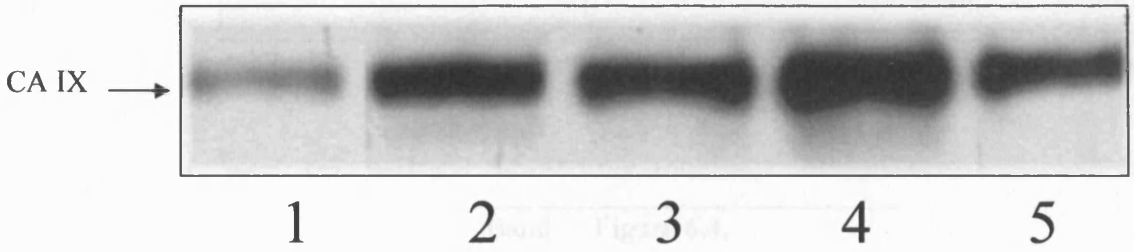
6.3.1. Background CA IX expression in A549 cells

A background expression of CA IX was observed in the absence of any treatment during or after serum starvation (see control lanes in all blots).

6.3.2. EGF and DFX dose response

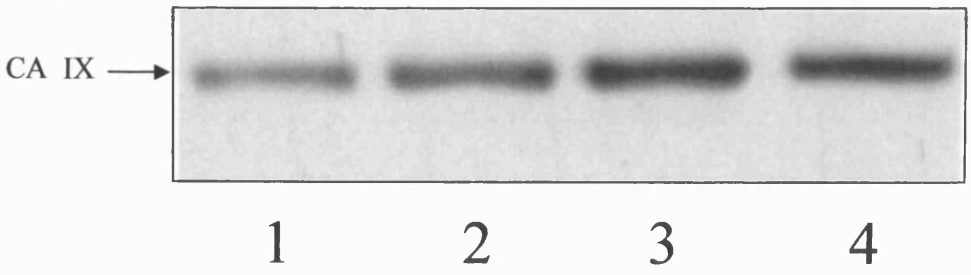
A dose response experiment found increasing CA IX expression in response to exposure to doses of 10ng/ml and above of EGF for 4 hours, treated after 24 hours serum starvation (figure 6.2). DFX induced CA IX at doses of 100µmol, 200µmol and 400µmol. Above 200µmol the level of induction of CA IX appeared to plateau (figure 6.3).

Figure 6.2. CA IX expression in A549 cells after treatment with increasing doses of EGF for 4 hours following 24 hours serum starvation



Lane	EGF dose figure 6.2. (ng/ml)	DFX dose figure 6.3. (µmol)
1	control	control
2	10	100
3	20	200
4	50	400
5	100	

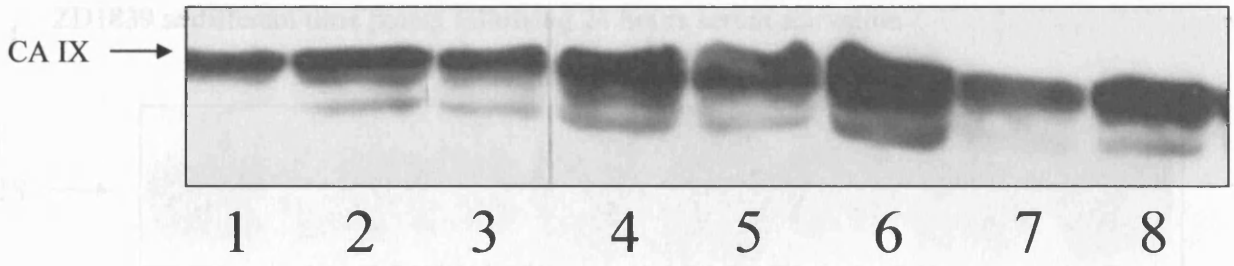
Figure 6.3. CA IX expression in A549 cells after treatment with increasing doses of DFX for 4 hours following 24 hours serum starvation



6.3.3. EGF time course

Initial time courses treating cells with 20ng/ml EGF after 24 hours serum starvation demonstrated a modest increase in CA IX expression following 1, 4 and 8 hours (figure 6.4.).

Figure 6.4. CA IX expression in A549 cells treated with 20ng/ml EGF at differing time points following serum starvation for 24 hours

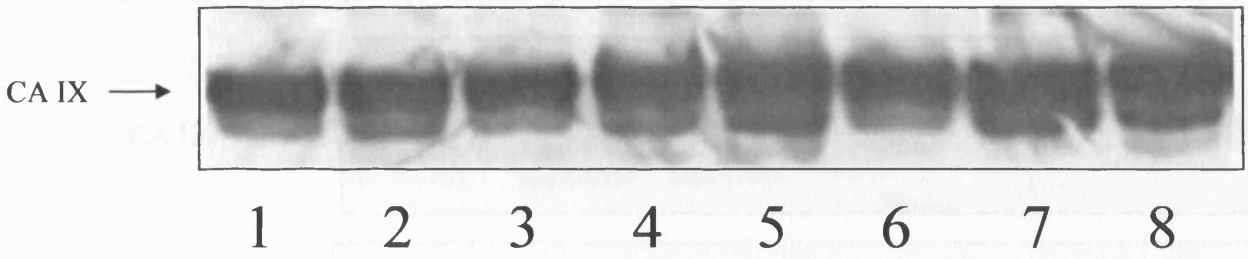


Band	Figure 6.4.
1	Control 30mins
2	EGF 20ng/ml 30 mins
3	Control 1 hour
4	EGF 20ng/ml 1 hour
5	Control 4 hours
6	EGF 20ng/ml 4 hours
7	Control 8 hours
8	EGF 20ng/ml 8 hours

6.3.4. Inconsistency of time course experiments

Repetition of the time course experiments failed to consistently reproduce the induction of CA IX with either DFX or EGF (figures 6.5. and 6.6.). Five micromolar ZD1839 did not suppress baseline CA IX expression at 4 and 8 hours post treatment (figure 6.5.).

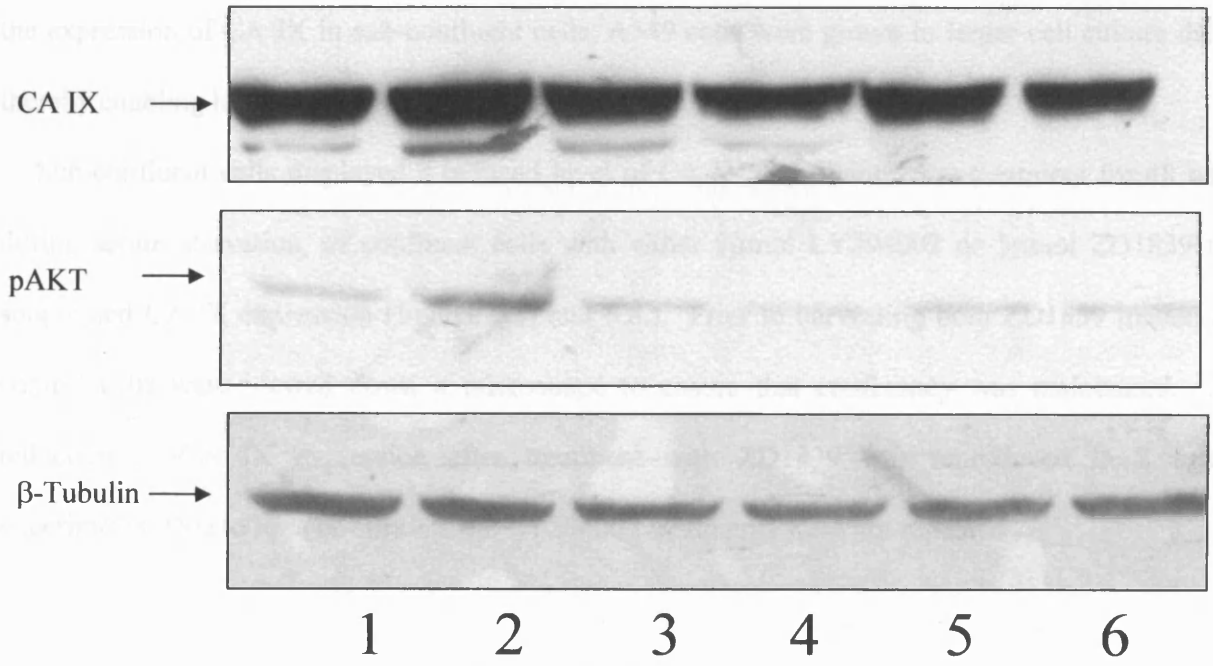
Figure 6.5. CA IX expression in A549 cells treated with 20ng/ml EGF or 200µmol DFX or 5µmol ZD1839 at different time points following 24 hours serum starvation



Band	Figure 6.5.
1	Negative control 4 hours
2	20ng/ml EGF 4 hours
3	200µmol DFX 4 hours
4	5µmol ZD1839 4 hours
5	Negative control 8 hours
6	20ng/ml EGF 8 hours
7	200µmol DFX 8 hours
8	5µmol ZD1839 8 hours

The time course experiment was repeated. The blot was initially probed for CA IX and again no induction was observed. The blot was then stripped and probed for phospho (p)Akt to check that the 20ng/ml EGF treatment was stimulating activity in the PI-3K pathway. Elevation of pAkt was seen at 1 hour. The blot was stripped a second time and probed for a housekeeper protein, β-tubulin to confirm correct protein loading of lanes (figure 6.6.).

Figure 6.6. CA IX, pAKT and β-tubulin expression in A549 cells treated with 20ng/ml EGF at differing time points following serum starvation for 24 hours



Lane	20ng/ml EGF
1	30 minutes
2	1 hour
3	2 hours
4	4 hours
5	8 hours
6	24 hours

6.3.5. Attempts to minimise background CA IX staining

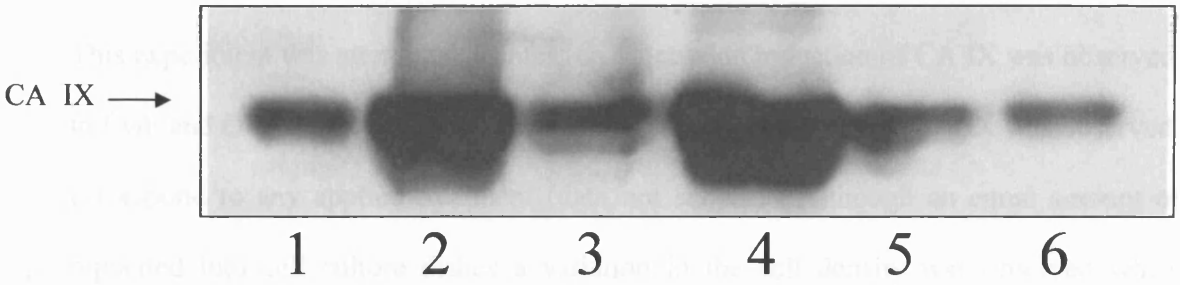
Serum starvation was prolonged to 48 hours to minimise background CA IX expression to exclude the possibility that modest induction of CA IX was being masked. Convincing induction of CA IX was still not observed in response to 20ng/ml EGF or 200μmol DFX.

6.3.6. Decreased cell density and prolonged treatment with ZD1839, an EGFR tyrosine kinase inhibitor, and LY294002, a PI3K-specific inhibitor

Increased cell density has previously been reported to induce CA IX expression. To investigate the expression of CA IX in sub-confluent cells, A549 cells were grown in larger cell culture dishes thereby enabling harvesting of cells prior to confluence.

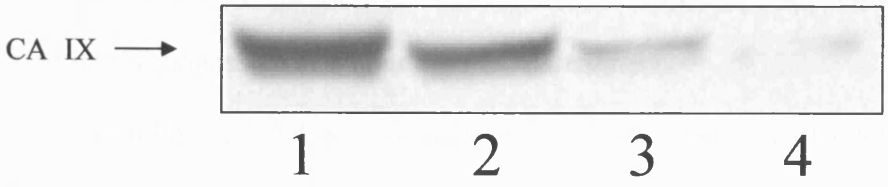
Sub-confluent cells displayed a reduced level of CA IX expression. Pre-treatment for 48 hours during serum starvation, of confluent cells with either 5μmol LY294002 or 5μmol ZD1839 also suppressed CA IX expression (figures 6.7. and 6.8.). Prior to harvesting both ZD1839 treated and control cells were viewed down a microscope to ensure that confluency was maintained. The reduction in CA IX expression after treatment with ZD1839 was reproduced in 2 further experiments. Due to time constraints the LY294002 treatments were not repeated

Figure 6.7. CA IX expression in sub-confluent and confluent A549 cells with and without treatment with 5μmol ZD1839 during serum starvation for 48 hours



1	DMSO control sub-confluent
2	DMSO control confluent
3	Negative control subconfluent
4	Negative control confluent
5	ZD1839 control sub-confluent
6	ZD1839 control confluent

Figure 6.8. CA IX expression in A549 confluent cells treated with LY20094 and sub-confluent cells during serum starvation for 48 hours



1	Negative control confluent
2	DMSO control confluent
3	LY294002 confluent
4	Sub-confluent cells

6.3.7. Treatment of subconfluent cells with DFX and EGF

Sub-confluent cells were treated with 20ng/ml EGF and 200μmmol DFX. As these cells did not have maximal CA IX expression it was postulated that both EGF and DFX would induce CA IX. This experiment was attempted 3 times; on 1 occasion induction of CA IX was observed in response to EGF and DFX. On the other 2 occasions a variable induction of CA IX was observed that did not correspond to any applied treatment (data not shown). Although an equal amount of cells were pippetted into cell culture dishes a variation in the cell density was observed when cells were harvested. Again time constraints prevented these problems from being addressed further.

6.4. DISCUSSION

The experiments outlined in this chapter have demonstrated that increased cell density induces CA IX expression and that this induction is suppressed by prolonged treatment (48 hours) of confluent A549 cells with ZD1839 and LY294002. Treatment of confluent cells with ZD1839 for shorter periods (4 and 8 hours) had no impact on CA IX expression. Treatments of confluent and sub-confluent A549 cells with EGF failed to consistently induce expression of CA IX. This was not due to investigator error as activation of the PI-3K pathway was achieved as demonstrated by induction pAKT expression at 1 hour after EGF treatment.

The 20ng/ml dose of EGF was selected in spite of the increased induction of CA IX expression seen with higher doses as previous studies had shown that this dose was sufficient to induce HIF-1α expression in tumour cell lines [278]. Furthermore higher doses of EGF have been shown to generate hydrogen peroxide molecules that inhibit tyrosine phosphatases. Such doses of EGF may result in activity of downstream PI-3K and MAPK pathways independently of EGF-EGFR binding [483, 484].

DFX is a hypoxia mimetic as it inhibits HIF-1α prolyl and asparagine hydroxylation thereby stabilizing and activating this transcription factor by a similar process to that of hypoxia. As such DFX has been shown to induce HIF-1α expression and transcriptional activity [186, 468, 485]. Surprisingly DFX failed to consistently induce CA IX expression. Although this appears to be at odds with the published data on HIF-1α there are no reports that DFX induces CA IX specifically and raises the possibility that processes other than hypoxia dependent inhibition of hydroxylation is required for CA IX induction.

The inhibitory effects of prolonged treatment with ZD1839 and LY294002 suggest that under confluent conditions CA IX is induced either directly or indirectly by activation of the EGFR/ PI-3K pathway. Due to time constraints the experiment demonstrating suppression of CA IX induction by the LY294002 could not be repeated however this single experiment is in agreement with previous reports in HeLa cells [281]. The lack of effect of ZD1839 at 4 and 8 hours suggests the influence of EGFR activation on CA IX expression is indirect. Experiments in our laboratory have shown growth arrest achieved by ZD1839 is not apparent until 48 hours post treatment in A549 cells. Such growth arrest may reduce O_2 consumption and hence hypoxia thereby indirectly reducing CA IX expression.

The failure of EGF to consistently induce CA IX in confluent A549 cells suggests that the regulatory mechanisms inhibited by ZD1839 are maximally induced under confluent conditions. A549 cells are known to secrete growth factors such as transforming growth factor (TGF)α and insulin-like growth factor (IGF)-1 both of which can activate the PI-3K pathway and hence possibly HIF-1α. The activity of these factors may increase CA IX expression as cell density increases again in a direct or indirect fashion [486-488]. The proposal that the increased CA IX expression is a result of an indirect effect created by an increase in relative hypoxia is supported by the observation that the induction of CA IX by increased cell density is abrogated by gentle stirring [281].

In an attempt to prove the lack of response to EGF and DFX was due to existing maximal CA IX expression in confluent cells, sub-confluent cells were treated with DFX and CA IX. Initially shorter incubation times were used to try and harvest cells prior to confluency but these were frustrated by the apparent exponential increase in the rate of cell growth as cells approached confluency. To enable harvesting of sub-confluent cells larger plates were used. Sub-confluent cells consistently expressed a reduced level of CA IX. These cells were treated with EGF and DFX but again no consistent induction of CA IX was observed compared to control. Difficulties were encountered when attempting to harvest sub-confluent cells at a uniform density as cells in different culture dishes grew at marginally different rates. In view of the sensitivity of CA IX to increasing cell density the changes in CA IX expression could not be attributed to the treatments applied.

In summary, these findings applied with the associations between EGFR and CA IX expression reported in chapter 4 imply either a direct or indirect causative mechanism exists whereby EGFR activity induces CA IX expression. In future studies the suppression of CA IX by the LY294002 needs to be confirmed. The regulation of CA IX under confluent conditions needs to be further explored. Treatment of confluent A549 cells with neutralising antibodies to TGF alpha and IGF-1 would be expected to suppress CA IX expression if these factors are acting in an autocrine fashion. EGF may reverse the effects of these antibodies if the aforementioned postulate holds true. Further experiments using subconfluent cells would be of dubious use as it is debatable as to whether the behavior of these cells bears any resemblance to how tumour cells behave *in vivo*.

7.1. FINAL SUMMARY

The initial hypothesis was that hypoxia was an important factor in the pathogenesis of NSCLC; as a consequence markers of hypoxia were likely to be of prognostic value. The initial objective of this thesis was to optimise immunohistochemical assays for HIF-1α, HIF-2α and CA IX in NSCLC. Reliable and reproducible assays have been described for HIF-1α and CA IX. Unfortunately problems were not resolved with the HIF-2α assay and doubts remain concerning reproducibility. However the method used has successfully been employed in breast cancer and meaningful results published by Leek *et al.* [151]. The inter-relationships of these hypoxia related factors with other clinicopathological and biological factors and the prognostic implications have been studied.

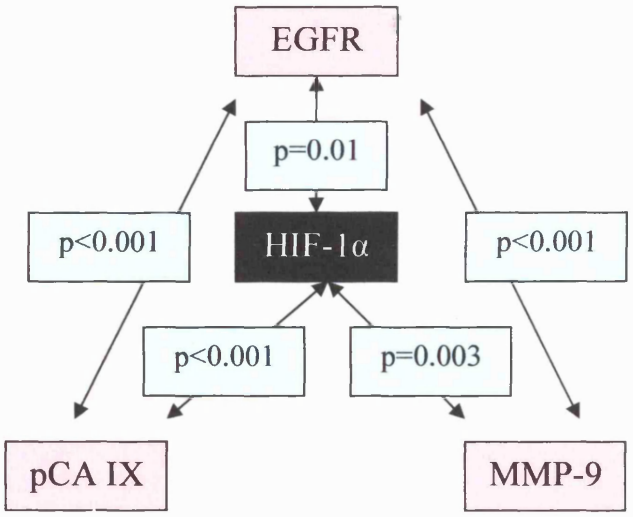
During preliminary reading I noted that little had been reported on the presence of TN in tumour sections with regard to the biological behaviour of NSCLC and patient prognosis. This was in stark contrast to a number of other solid tumours, especially breast cancer where TN is recognised as an important prognostic factor. Dr. Louise Jones and myself devised a simple scoring system. The presence of extensive TN was found to be an independent poor prognostic factor, in keeping with the initial hypothesis. Associations were found between the extent of TN and platelet count, T-stage and p53 expression and a near significant association was found with MMP-9 expression. The possible mechanisms behind these associations have been discussed in chapter 3.

The different patterns of CA IX staining have been described in chapter 4. The spatial relationships of CA IX positive tumour cells and tumour cell clusters with microvessels and associations of CA IX expression with TN add further weight to the contention that CA IX may be used as a marker of hypoxia in solid tumours. Before CA IX can be used in other solid tumour types similar studies need to be conducted in the tumour type selected. This was evidenced by work in colorectal cancer by our group where although CA IX was associated with a poor prognosis no association was found with the presence of TN. This suggests that factors other than hypoxia may

be involved in the regulation of CA IX. Indeed a close association between CA IX expression and EGFR was found. The possible pathways by which EGFR signalling may influence CA IX expression are discussed in chapter 4. The aforementioned postulate is further strengthened by finding that pCA IX expression was associated with a poor prognosis but not with the extent of TN.

HIF-1α expression was found to be closely associated with CA IX expression and EGFR expression. These results gave additional confidence to the methods used, as CA IX is a transcriptional target of HIF-1α and therefore a close association would be expected between the expressions of these 2 factors. Positive HIF-1α was not associated with prognosis but high HIF-1α expression was. Using HIF-1α as a continuous variable a near significant association was found with prognosis. The change in the prognostic data depending on the HIF-1α cut point used to dichotomise the series may reflect increasingly hypoxic environments in those cases with equal to or greater than 60% HIF-1α expression. HIF-1α expression was also found to be associated with T-stage, TN, p53 and MMP-9 expression (see figure 7.1.). The possible mechanisms responsible for these associations have been discussed in chapter 5.

Figure 7.1. Inter-relationships between HIF-1α, EGFR, CA IX and MMP-9



The associations between EGFR and CA IX expression and the prognostic ramifications for patients displaying these patterns of expression prompted me to study the relationship between these 2 factors *in vitro*. The working hypothesis was that EGFR related signalling may induce HIF-1α expression and activity and thereby upregulate CA IX. To study this an EGFR positive cell line was treated with EGF. No consistent induction of CA IX was measured using western blotting techniques although expression was suppressed by prolonged exposure to ZD 1839. Increased cell density was seen to consistently induce CAIX expression. The conclusions drawn were that EGFR signalling was unlikely to be directly responsible for increased CA IX expression. However EGFR related signalling may indirectly induce CA IX by stimulating tumour cell proliferation and thereby increasing tumour cell density. The relative hypoxia caused by increased cell density may induce CA IX expression. Alternatively autocrine signalling stimulating downstream EGFR related pathways may have masked any CA IX induction by EGF treatment. Further work is therefore required to exclude this as a possibility.

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Tumour necrosis is an independent prognostic marker in non-small cell lung cancer: correlation with biological variables

Daniel E.B. Swinson [a], J. Louise Jones [b], Donna Richardson [a], Giles Cox [a], John G. Edwards [a], Kenneth J. O'Byrne [a,*]

[a] *Department of Oncology, University Hospitals of Leicester NHS Trust, Leicester LE1 5WW, UK*
[b] *Breast Cancer Research Unit Department of Pathology, University Hospitals of Leicester NHS Trust, Leicester LE1 5WW, UK*

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Abstract

Background: Tumour necrosis (TN) is recognized to be a consequence of chronic cellular hypoxia. TN and hypoxia correlate with poor prognosis in solid tumours. *Methods:* In a retrospective study the prognostic implications of the extent of TN was evaluated in non-small cell lung cancer (NSCLC) and correlated with clinicopathological variables and expression of epidermal growth factor receptor, Bcl-2, p53 and matrix metalloproteinase-9 (MMP-9). Tissue specimens from 178 surgically resected cases of stage I–IIIA NSCLC with curative intent were studied. The specimens were routinely processed, formalin-fixed and paraffin-embedded. TN was graded as extensive or either limited or absent by two independent observers; disagreements were resolved using a double-headed microscope. The degree of reproducibility was estimated by re-interpreting 40 randomly selected cases after a 4 month interval. *Results:* Reproducibility was attained in 36/40 cases, Kappa score = 0.8 $P < 0.001$. TN correlated with T-stage ($P = 0.001$), platelet count ($P = 0.004$) and p53 expression ($P = 0.031$). Near significant associations of TN with N-stage ($P = 0.063$) and MMP-9 expression ($P = 0.058$) were seen. No association was found with angiogenesis ($P = 0.98$). On univariate ($P = 0.0016$) and multivariate analysis ($P = 0.023$) TN was prognostic. *Conclusion:* These results indicate that extensive TN reflects an aggressive tumour phenotype in NSCLC and may improve the predictive power of the TMN staging system. The lack of association between TN and angiogenesis may be important although these variables were not evaluated on serial sections.

Keywords: Tumour necrosis; Matrix metalloproteinase-9; p53; Epidermal growth factor receptor; Platelet count

1. Introduction

Increased cellular hypoxia correlates with resistance to radiotherapy and chemotherapy, increased metastatic potential and a worse prognosis in solid tumours [1–3].

It is conventionally accepted that coagulative necrosis, a common feature of solid tumours, is caused by chronic ischaemic injury, which suggests the degree of tumour necrosis (TN) reflects the level of intra-tumour hypoxia [4]. Indeed exposure of cells in vitro to hypoxia induces necrosis [5].

TN has previously been reported as an indicator of a poor prognosis in non-small cell lung cancer (NSCLC) in a study of 593 patients who underwent surgical resection of their tumours with curative intent [6]. Furthermore, two smaller studies in stage 1 NSCLC and non-neuroendocrine large cell carcinoma have correlated TN with poor prognosis [7,8]. Studies in breast cancer, gastrointestinal stromal tumours, Ewing's sarcoma of the bone and renal carcinoma, have found similar results [9–12].

TN has been most extensively investigated in breast carcinoma. In ductal carcinoma in situ (DCIS) TN is an independent indicator of ipsilateral recurrence. In invasive carcinoma of the breast TN has been shown to correlate with increased tumour size, high-grade disease,

Abbreviations: NSCLC, non-small cell lung cancer; TN, Tumour necrosis; MMP-9, metalloproteinase-9; EGFR, epidermal growth factor receptor; VEGF, vascular endothelial growth factor; ER, oestrogen receptor; DCIS, ductal carcinoma in situ.

* Corresponding author. Tel.: +44-116-258-7602; fax: +44-116-258-7599

E-mail address: kobyrne@uhl.trent.nhs.uk (K.J. O'Byrne).

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microvessel density (MVD), macrophage infiltrates that express vascular endothelial growth factor (VEGF), negative oestrogen receptor (ER) status, decreased relapse free survival and a worse prognosis [13,9]. This suggests fast growing breast carcinomas create a hypoxic environment that results in TN and stimulates angiogenesis in part due to the release of angiogenic growth factors from infiltrating macrophages. The associations with high-grade, negative ER status and poor prognosis reflect an aggressive phenotype.

The purpose of this study was to evaluate the impact of TN on patient outcome in stage I–IIIA resected NSCLC. In addition, the relationship between TN and clinicopathological and biological parameters was evaluated.

2. Methods

One hundred and seventy eight specimens were studied from patients who had been operated on for stage I–IIIA NSCLC with curative intent between January 1991 and December 1996. The confounding variable of peri-operative mortality was minimised by only including patients surviving >60 days following surgery. Of the 178 patients evaluated, 125 (70%) were male and 53 (30%), female. The mean age at surgery was 64.7 years (S.D. 7.9, median 68). Positive resection margins had been treated with adjuvant radiotherapy in 14 patients and 1 patient had received adjuvant chemotherapy. The final staging was based on the findings at surgery and the histopathology report. Hospital notes of the patients were reviewed and if necessary the local cancer registry or patient's General Practitioner was contacted to collate pre-operative platelet counts and survival data. One hundred and twenty six patients had died at the time of analysis of which 110 had died of lung cancer.

The specimens studied were routinely processed, formalin-fixed and paraffin-embedded. Only blocks containing the advancing edge of the primary tumour were evaluated. Tissue sections of 4 μm thickness were cut onto glass slides that were previously treated with 2% 3-aminopropylethoxysilane (in methanol) and dried overnight at 37 °C to assist with section to slide adhesion. Slides were stained by submersion in haematoxylin for 15 s.

Sections were assessed at low magnification ($\times 40$) using light microscopy by two observers independently in a blinded fashion. Where a disagreement was recorded a consensus was determined using a dual headed microscope. JLJ, a consultant histopathologist, adjudicated unresolved differences. The degree of tissue necrosis on the section was scored 0, no necrosis; 1, 1 focus of necrosis per low power field (LPF), each focus $<10\%$ per LPF; 2, >1 focus of necrosis each occupying $<10\%$ or 1 focus of necrosis occupying 10 to $<30\%$ per LPF; 3, single or multiple areas of necrosis $>30\%$ per LPF. A mean of 10 LPF per section were evaluated depending on the size of the section (Fig. 1a–c). The median score was used as the cut point, which defined scores ≥ 3 as extensive and scores 0–2 as negative or limited.

Reproducibility of interpretation was tested 4 months after initial interpretation by randomly selecting and re-interpreting 40 cases. Agreement was found in 36/40 cases, which gave a Kappa score of 0.8 $P<0.001$.

The specimens had previously been evaluated for the expression of matrix metalloproteinase-9 (MMP-9), epidermal growth factor receptor (EGFR), Bcl-2, p53 and MVD. Standard immunohistochemical methods were employed using the anti-MMP-9 mouse monoclonal antibody ($n=169$) (Mab) 56-2A4 (Chemicon International Ltd, UK), anti-EGFR mouse Mab EGFR.113 ($n=179$) (Novocastra Laboratories Ltd, UK), anti-Bcl-2 mouse Mab clone 124 ($n=180$) (Dako, UK) and anti-p53 rabbit polyclonal antibody NCL-p53-CM1 ($n=180$) (Novocastra) and anti CD34

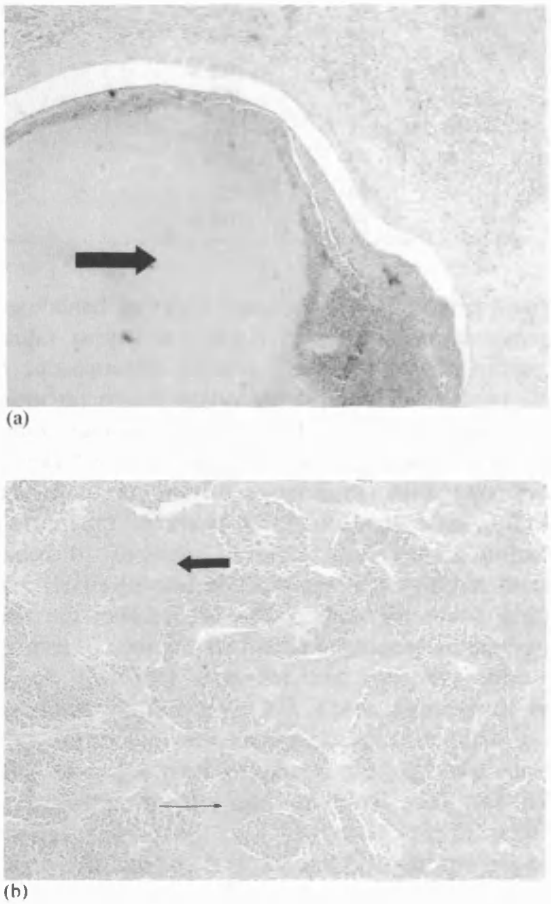

Fig. 1. Haematoxylin and eosin sections of NSCLC. (a) Block arrow: $\geq 30\%$ area of necrosis; (b) thin arrow: $<10\%$ focus of necrosis; block arrow: 10 to $>30\%$ focus of necrosis.

(Dako) respectively [14–16]. The MVD was assessed using the Chalkley counting method ($n = 176$) [15].

The SPSS software system (SPSS for Windows Version 9.0) was used for the statistical analysis. The Kappa test was used to test for the reproducibility of the interpretation. The χ^2-test was used to analyze the associations between categorical variables. Survival curves were plotted using the Kaplan–Meier method and log-rank test was used to assess the statistical significance of differences in outcome. The influence of clinicopathological and biological variables on overall survival was assessed using the Cox regression forward log-rank model. The entry criteria were $P \leqslant 0.05$ and removal criteria were $P \geqslant 0.1$ from the equation.

3. Results

Coagulative type TN was observed in the majority of cases. TN positively correlated with platelet count ($P = 0.004$), T-stage ($P = 0.001$) and p53 expression ($P = 0.031$). Trends towards associations were found with N-stage ($P = 0.063$) and MMP-9 expression ($P = 0.058$). No associations were found with tumour grade, EGFR or Bcl-2 expression (Table 2).

TN did not correlate with MVD with the cut point taken as $\geqslant$median ($P = 0.982$), the cut point taken as $\geqslant$top tertile ($P = 0.67$) or with the MVD divided into separate tertiles ($P = 0.31$). Subgroup analysis dividing the series into stage 1 ($P = 0.31$, $n = 87$), stage 2 ($P = 0.95$, $n = 50$) and stage 3 ($P = 0.053$, $n = 39$) also did not show a significant association between TN and MVD.

The survival data from previous studies was up-dated and statistical analysis repeated to include TN [15,16]. Univariate prognostic indicators include the clinicopathological variables TN ($P = 0.0016$), T-stage ($P = 0.033$), N-stage ($P = 0.0002$), gender ($P = 0.028$) and the platelet count ($P = 0.026$). MMP-9 was the only prognostic biological variable ($P = 0.023$) (Table 1) (Fig. 2).

Subgroup analysis in different tumour stages extensive TN was associated with a poor prognosis in early disease (stages I and II) ($P = 0.0014$, $n = 138$). In stage IIIA cases there was no association between TN and prognosis ($P = 0.54$, $n = 40$).

In multivariate analysis stage ($P < 0.0001$), TN ($P = 0.016$) and gender ($P = 0.031$) were independent prognostic markers (Table 3).

Table 1
Relationships between clinicopathological and biological parameters and TN

Prognostic factor		Low TN	High TN	χ^2 P value
Age	$\geqslant$ Median	55	34	0.36
	< Median	49	40	
Gender	Male	73	52	0.99
	Female	31	22	
Histology	Adenocarcinoma	32	17	0.20
	Large	8	6	
	Squamous	60	51	
	Other	4	0	
Grade (differentiation)	Well/moderate	54	37	0.80
	Poor	50	37	
T-Stage	1	27	4	0.001
	2	69	57	
	3	8	13	
N-Stage	0	60	33	0.063
	1	25	30	
	2	19	11	
Platelet count	< Median	59	24	0.004
	$\geqslant$ Median	44	45	
EGFR	< 20%	50	35	0.87
	$\geqslant$ 20%	53	39	
MMP-9	< 20%	51	28	0.058
	$\geqslant$ 20%	44	44	
Bcl-2	+ve	42	30	0.98
	−ve	62	44	
p53	< 20%	62	32	0.031
	$\geqslant$ 20%	42	42	

4. Discussion

The results demonstrate that extensive TN correlates with poor survival. This suggests a paradoxical relationship whereby evidence of increased tumour cell death indicates a more aggressive cancer. This relationship can be explained by rapid tumour growth outstripping the vascular supply creating a hypoxic microenvironment and subsequently causing TN. The brittle nature of tumour microvasculature predisposes to vascular insufficiency exacerbating this effect [17–19]. Such a relationship has been proposed to exist in breast cancer [9]. These findings are in agreement with two recent important studies investigating the expression of factors induced by hypoxia in NSCLC. Hypoxia inducible factor (HIF)-1α and HIF-2α are transcription factors, which are induced by and orchestrate many cellular responses to hypoxia, including angiogenesis, up-regulation of glycolytic enzymes and possibly improves intracellular buffering [20,21]. The expression of both factors correlates with angiogenic growth factors, angiogenesis and a poor prognosis although the correlation between HIF-1α and prognosis did not reach statistical significance [21]. Carbonic anhydrase (CA) IX is up-regulated by HIF-1α and HIF-2α and has been proposed to be an immunohistochemical marker of hypoxia. Expression of CA IX has also been demonstrated to be associated with increased angiogenesis and a poor prognosis in NSCLC [20].

Table 2
Prognostic significance of clinicopathological and biological parameters

Prognostic factor		*n*	Hazard ratio	95% CI	*P*
Gender	Female	53	1		
	Male	125	1.58	1.05–2.37	0.028
Histology	Squamous	111	1		
	Large	14	1.21	0.8–1.82	
	Adenocarcinoma	49	0.89	0.41–1.92	0.70
	Other	4			
Grade	Well/mod diff	91	1		
	Poor diff	87	1.15	0.81–1.64	0.42
Stage	I	86	1		
	II	52	1.94	1.29–2.92	
	IIIA	40	2.53	1.63–3.94	0.0001
Platelet count	< Median	83	1		
	⩾ Median	89	1.51	1.05–2.16	0.026
TN	Score 0–2	104	1		
	Score 3–5	74	1.76	1.24–2.50	0.0016
EGFR	< 20%	92	1		
	⩾ 20%	85	1.02	0.72–1.46	0.91
MMP-9	< 20%	88	1		
	⩾ 20%	79	1.53	1.06–2.20	0.023
Bcl-2	−ve Bcl-2	106	1		
	+ve Bcl-2	72	0.81	0.56–1.16	0.25
p53	+ve	62	1		
	−ve	42	1.12	0.79–1.60	0.51

No association was found between MVD and the degree of TN either in the entire series or in subgroup analysis when the series was divided into subgroups according to the stage of disease. The lack of an association may reflect an importance difference between NSCLC and breast cancer. However the fact that TN and MVD was not assessed on serial sections is an important caveat as there is a high degree of variability in the pattern of angiogenesis through out NSCLC tumours, which may mask such an association [22]. The extent of TN was not assessed on the slides that the MVD was evaluated, as this would have introduced a degree of bias that would negate any meaningful result. The association between (CA) IX and angiogenesis that was evaluated in serial sections, adds weight to the contention that the result found in this study may not be a true reflection of the biological processes governing angiogenesis in NSCLC [20]. Alternatively although chronic hypoxia results in TN, TN can also be secondary to acute infarction. Acute infarction may be a relatively common occurrence in the tumour environment due to the brittle and relatively inconsistent nature of the tumour microvasculature [17–19].

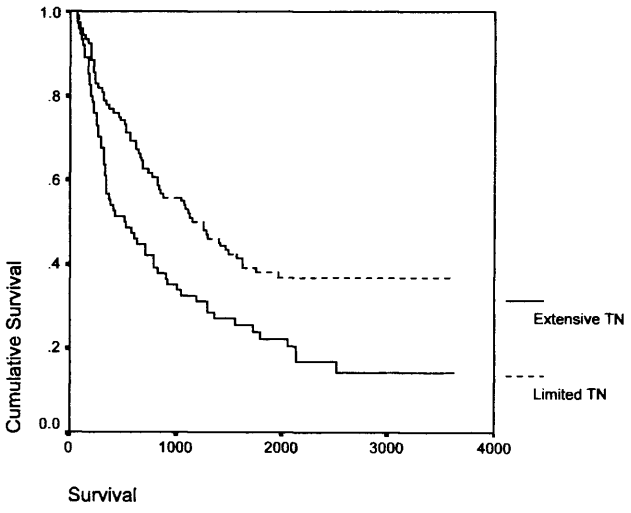

Fig. 2. Kaplan–Meier survival curve for TN in NSCLC.

Table 3
Multivariate analysis

Factor	Variable	Hazard ratio	95% CI	*P*-value
Stage	1	1.00		
Nodal status	2	1.67	1.08–2.62	
	3a	2.32	1.45–3.70	0.002
Gender	Female	1.00		
	Male	1.61	1.04–2.50	0.031
TN	Limited	1.00		
	Extensive	1.59	1.09–2.32	0.023

The association with T-stage adds weight to the proposal that TN reflects rapid local growth of NSCLC tumours. The contention that the extent of TN within the primary tumour reflects aggressive invasive disease is supported by the trends towards an association with N-stage and tumour cell MMP-9 expression. MMP-9 digests the extracellular matrix enhancing the invasive potential and facilitates angiogenesis; increased expression is a univariate indicator of a worse prognosis in NSCLC [16]. In support of this observation an association between MMP-9 and TN has been reported in breast cancer [23].

Interestingly relationship between TN and patient outcome is lost in the stage III subgroup. A similar finding has previously been reported in a series of 30 patients treated with concurrent platinum based chemotherapy and radiotherapy. This may be due to the small size of both this and our patient samples or may reflect a real reduction in the discriminatory value of this marker in later stage disease [24].

In non-malignant cell lines hypoxia induces apoptosis and up-regulation of p53 has been shown to have a key role in this process [25]. Persistent hypoxia mediates selection for mutant p53 as cells expressing wild type p53 undergo programmed cell death [26]. As wild type p53 has a short half-life immunostaining tends to detect overexpression of mutant p53 [27]. The association between p53 expression and TN may reflect both the selection of mutant p53 and the up-regulation of wild type p53 in non-genetically mutated cells. The lack of an association between p53 expression and outcome suggests a degree of heterogeneity exists in the population of tumour cells, some overexpressing wild type p53 and others expressing mutant p53. In breast the presence of p53 positivity and absence of Bcl-2 correlate with TN [28] [29]. Both our observations and those of Tormanen et al. show only the relationship between p53 and TN is repeated in NSCLC [30].

The association between platelet count and TN may simply reflect the fact that both are seen in tumours with an aggressive phenotype NSCLC [15]. However vascular stasis, exposure of the subendothelial matrix and increased expression of platelet binding factors, which are increased in tumours may lead to increased platelet adhesion and activation. In turn, activation of platelets could result in the formation of platelet plugs obstructing the vascular supply causing further TN [31,32]. TN may also result in thrombocytosis through hypoxic induction of interleukin (IL)-6 [33]. IL-6 is a potent stimulator of platelet synthesis and is widely expressed in NSCLC [34,35].

A possible criticism of the study is that the degree of TN was assessed subjectively. The high degree of reproducibility that was achieved when the specimens were interpreted 4 months apart demonstrates the reliability of this method. Furthermore, previous published data investigating TN have used similar techniques [9].

Appropriate work for future serial tissue section studies would be to evaluate the associations between TN and MVD, VEGF expression, markers of tumour hypoxia such as CA IX and cytokine expression by infiltrating inflammatory cells [32,36]. Tumour cavitation may be increased in necrotic tumours. A study investigating whether TN and tumour cavitation correlate may enable additional radiological evidence of the biology of the tumour to be gained prior to treatment.

In conclusion we have demonstrated that extensive TN is an independent poor prognostic factor in NSCLC and may represent an easily measurable parameter, which adds to the conventional staging of this tumour type. The positive correlations with T-stage and platelet count and the trend towards associations with N-stage and tumour cell MMP-9 expression indicate that TN is associated with an aggressive NSCLC phenotype in early disease.

Acknowledgements

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Carbonic Anhydrase IX Expression, a Novel Surrogate Marker of Tumor Hypoxia, Is Associated With a Poor Prognosis in Non–Small-Cell Lung Cancer

By Daniel E.B. Swinson, J. Louise Jones, Donna Richardson, Charles Wykoff, Helen Turley, Jaromir Pastorek, Nick Taub, Adrian L. Harris, and Kenneth J. O'Byrne

Purpose: **To evaluate carbonic anhydrase (CA) IX as a surrogate marker of hypoxia and investigate the prognostic significance of different patterns of expression in non–small-cell lung cancer (NSCLC).**

Methods: **Standard immunohistochemical techniques were used to study CA IX expression in 175 resected NSCLC tumors. CA IX expression was determined by Western blotting in A549 cell lines grown under normoxic and hypoxic conditions. Measurements from microvessels to CA IX positivity were obtained.**

Results: **CA IX immunostaining was detected in 81.8% of patients. Membranous (m) ($P = .005$), cytoplasmic (c) ($P = .018$), and stromal ($P < .001$) CA IX expression correlated with the extent of tumor necrosis (TN). The mean distance from vascular endothelium to the start of tumor cell positivity was 90 μm, which equates to an oxygen pressure of 5.77 mmHg. The distance to blood vessels from individual tumor cells or tumor cell clusters was greater if they expressed mCA IX than if they did not ($P < .001$). Hypoxic exposure of A549 cells for 16 hours enhanced CA IX expression in the nuclear and cytosolic extracts. Perinuclear (p) CA IX ($P = .035$) was associated with a poor prognosis. In multivariate analysis, pCA IX ($P = .004$), stage ($P = .001$), platelet count ($P = .011$), sex ($P = .027$), and TN ($P = .035$) were independent poor prognostic factors.**

Conclusion: **These results add weight to the contention that mCA IX is a marker of tumor cell hypoxia. The absence of CA IX staining close to microvessels suggests that these vessels are functionally active. pCA IX expression is representative of an aggressive phenotype.**

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INCREASED TUMOR cell hypoxia, measured with a polarographic needle, correlates with increased metastatic potential, resistance to radiotherapy, and a worse prognosis in solid organ tumors, including non–small-cell lung cancer (NSCLC).[1-3]

Carbonic anhydrase (CA) IX is upregulated by hypoxia and has a predominantly perinecrotic pattern of tumor cell expression.[4-6] In serial sections taken from biopsies of basal and squamous cell skin and bladder transitional cell carcinomas, a high degree of overlap was found between CA IX and the recognized hypoxic marker pimonidazole.[7-10] A prospective study found a close correlation between polarographic measurements of intratumoral oxygen tensions and CA IX immunostaining in cervical carcinoma.[11] These findings indicate that CA IX expression may be used as a surrogate marker of cellular hypoxia.

CA IX previously has been observed in 80% of resected NSCLC. In bronchial biopsies, negative CA IX expression distinguished preneoplastic lesions from carcinoma-in-situ and microinvasive disease.[12] CA IX has been proposed as a tumor marker for renal cell carcinoma and as a biomarker of cervical dysplasia and neoplasia in Papanicolaou smears.[13,14] In invasive breast cancer, increased expression of CA IX was associated with markers of an aggressive phenotype and a worse outcome.[4,6]

CA IX is regulated by the hypoxia-inducible factor (HIF)-1 transcription factor.[10] HIF-1 is composed of two subunits, HIF-1 alpha and HIF-1 beta. HIF-1 is stabilized by hypoxia, and activates target genes that contain a hypoxic response element in their promoter region.[15] Increased expression of HIF-1 and the related transcription factor HIF-2 have been implicated in tumorigenesis in NSCLC.[16] In this study, we examined the prognostic implications and patterns of expression of CA IX in NSCLC.

PATIENTS AND METHODS

Study Population

The tissue specimens evaluated were taken from 204 consecutive patients with histologic stage I to IIIA NSCLC who had undergone resection of NSCLC with curative intent. Twenty-four patients who died within 61 days of surgery were excluded from the study to reduce the confounding variable of perioperative mortality; five more patients were excluded because of lack of available tissue. The 30-day mortality rate was 6.4%, which is marginally higher than the internationally published 30-day mortality rate (3.9%).[17] Of the 175 stained specimens, 125 were from males and 50 were from females. The mean age at surgery was 64.8 years (SD, 8.0; range, 33.8 to 79.1). Positive resection margins were found in 15 specimens (8.5%), the presence of which was associated with stage IIIA disease (n = 8 of 15, $P = .011$). One patient received adjuvant chemotherapy, and of the 15 patients who were treated with adjuvant radiotherapy, 10 had stage IIIA, six had stage II, and one had stage I disease. The final staging was based on the findings at surgery and the histopathology report. Hospital notes of the patients were reviewed and, if necessary, the local cancer registry or patient's general practitioner was contacted to complete follow-up. From these data it was established that 124 patients (70.5%) had died by the time of analysis and, of these deaths, 18 (10.2%) were not cancer related.

From the Departments of Oncology, Pathology, and Epidemiology, University Hospitals of Leicester NHS Trust; Imperial Cancer Research Fund, Molecular Oncology Unit, Institute of Medicine, John Ratcliffe Hospital, Oxford, United Kingdom; and Institute of Virology, Slovak Academy of Sciences, Slovak Republic.

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Address reprint requests to Kenneth J. O'Byrne, MD, University Hospitals of Leicester NHS Trust, Leicester LE1 5WW, United Kingdom; email: ken.obyrne@uhl-tr.nhs.uk.

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Table 1. Immunohistochemistry Techniques and Antibodies Used on Serial Sections to Determine Stromal Cell Type Expressing Cartonic Anhydrase IX

Antibody	Manufacturer	Cell Type Marker	Antigen Retrieval	Primary Antibody Dilution	Visualization Technique
Smooth muscle actin	Dako	Activated fibroblast	Nil	1:400	ABC + DAB
Vimentin	Dako	Fibroblast	0.1% Trypsin 10 minutes at 37°C	1:50	ABC + DAB
CD34	Novacastra	Endothelial cell	Nil	1:50	ABC + DAB
CD34 Double staining	Novacastra	Endothelial cell	Nil	1:50	APAAP + fuchsin
CD31	Dako	Endothelial cell	0.1% Trypsin 10 minutes at 37°C	1:50	ABC + DAB

Abbreviations: ABC, avidin biotin complex; DAB, diaminobenzidine substrate; APAAP, alkaline phosphatase antialkaline phosphatase.

MATERIALS

The specimens studied were routinely processed, formalin-fixed, and paraffin-embedded. Only blocks containing the advancing edge of the primary tumor were evaluated. Tissue sections of 4 μm thickness were cut onto glass slides that were previously treated with 2% 3-aminopropylethoxysilane (in methanol) and dried overnight at 37°C to assist with section-to-slide adhesion.

The murine monoclonal antibody (Mab) M75 recognizing the *N*-terminal domain of MN/CA IX protein has been previously described by Pastorekova et al.[18] The specificity of the monoclonal antibody M75 for CA IX was confirmed by Western blots and immunostaining of COS-7 cells transfected with CA IX cDNA.[19] The secondary antibody was rabbit antimouse polymer from the Envision kit (Dako, Ely, United Kingdom).

Immunohistochemistry

No antigen retrieval was necessary. Sections were dewaxed in xylene and rehydrated by passage through graded alcohols. Endogenous peroxidase was blocked by applying 0.03% hydrogen peroxidase containing sodium azide from the Envision kit (Dako) for 10 minutes. The application of 100 μL of 10% normal human serum for 15 minutes blocked nonspecific staining. One hundred microliters of M75 of working solution, which was diluted stock solution 1:50 (vol/vol) in 5% normal human serum, was applied for 30 minutes. Secondary rabbit antimouse polymer from the Envision kit (Dako) was applied for 30 minutes at room temperature. Visualization of CA IX was achieved by applying diaminobenzidine substrate (DAB) for 5 to 8 minutes. Sections were immersed in Tris-buffered saline for 5 minutes between steps. After DAB administration, slides were immersed under running tap water for 5 minutes; sections were then counterstained with hematoxylin, dehydrated by reverse passage through graded alcohols, and mounted using DPX (BDH Chemicals, Ltd., Poole, England).

Investigation of Stromal Cell Type Expressing CA IX

A panel of recognized immunohistochemical markers for vascular endothelium and myofibroblasts was used to stain serial sections cut from selected specimens with extensive stromal expression to investigate which stromal cell type was responsible for this pattern of positivity. The staining methods for each marker are summarized in Table 1.

Interpretation of CA IX Staining and Tumor Necrosis

Sections were assessed using light microscopy in a blinded fashion by two observers (D.E.B.S. and D.R.). If discrepancies were found, a consensus was reached using a conference microscope. Persistent discrepancies were adjudicated by a third observer (J.L.J). The percentage of tumor cells with membranous (m) CA IX expression and the percentage of tumor cells with cytoplasmic (c) expression were estimated. For subgroup analysis, mCA IX expression was divided into quartiles depending on the percentage of cells stained: negative, 0%; low, less than 5%; moderate, ≥ 5% to less than 30%; and extensive, ≥ 30%. Perinuclear (p) CA IX expression was classified as positive or negative. There were three distinct patterns classified as pCA IX expression: (1) staining obscuring the nuclear structures, (2) circumferential staining of the nuclear margins, and (3) discrete areas of staining adjacent to and indenting the nucleus (Fig 1). Stromal expression was assigned a score of 0 to 3 (0 = no stromal expression, 1 = occasional stromal expression, 2 = moderate stromal expression, and 3 = extensive stromal expression). The

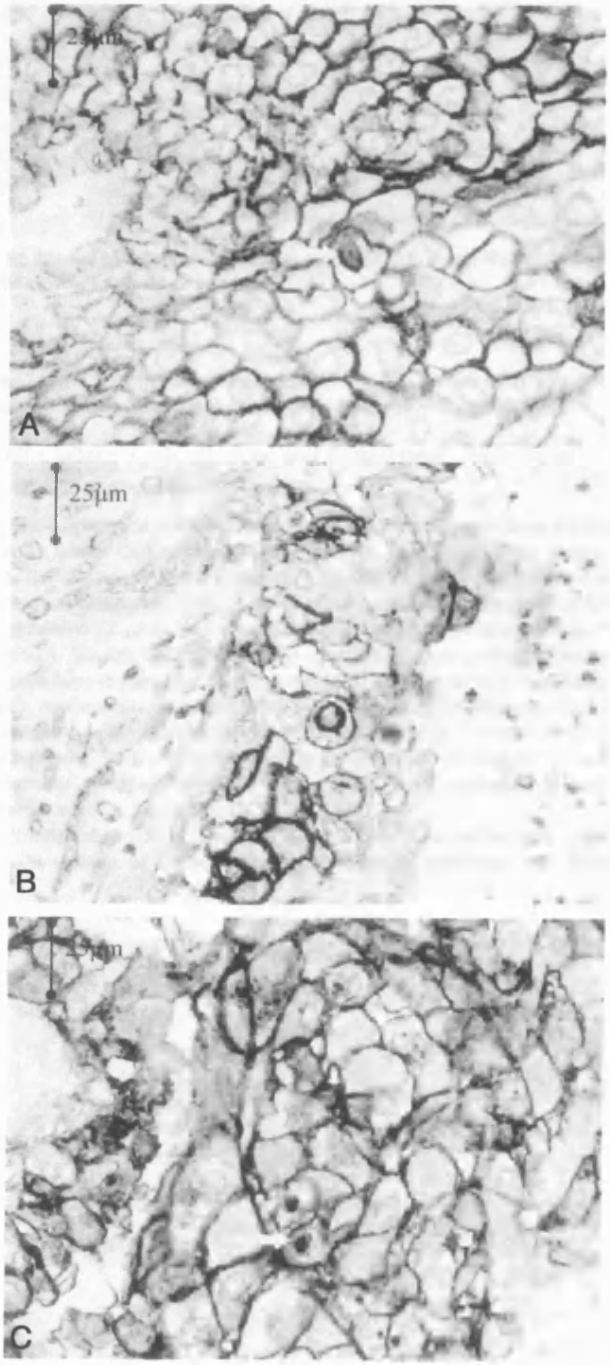

Fig 1. Three sections of non–small-cell lung cancer stained with M75 (magnification × 400) demonstrating different perinuclear carbonic anhydrase IX staining. (A) Type 1, staining obscuring the nucleus; (B) type 2, circumferential staining around the nucleus; and (C) type 3, a discrete area of staining indenting the nucleus.

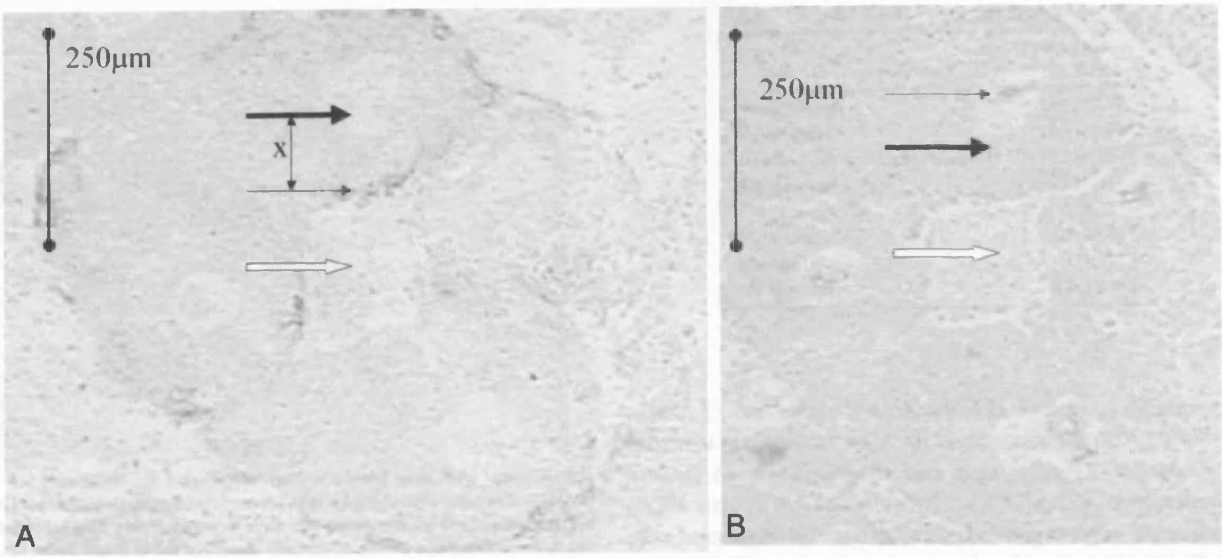

Fig 2. Non–small-cell lung cancer cells (block arrows) stained with M75 (thin arrow, Fig 2A) and endothelium stained with anti-CD34 (thin arrow, Fig 2b). Arrow "x" marks the distance from endothelium to carbonic anhydrase IX-positive tumor cells. White arrow labels tumor necrosis.

degree of tumor necrosis (TN) on the section was assessed by two independent observers and was scored as either limited or extensive.[20]

Estimation of the Oxygen Pressure (Po_2) at Which mCA IX Positivity Starts

To assess at what distance from blood vessels mCA IX staining commenced, three specimens were chosen that had extensive expression. Serial sections were stained for CD34 and CA IX, as described above. Measurements were only taken from blood vessels that were surrounded by tumor cells with positivity, so that three distances could be measured from each vessel in different directions. In addition, blood vessels that had been cut through an oval or longitudinal plain were avoided. In total, six blood vessels were suitable, and 18 measurements were made (Fig 2). The distance from the CD34 staining endothelium to mCA IX–positive tumor cells was measured using a graticule with a slide wire micrometer, calibrated with a calibration slide by D.E.B.S. This method previously was described by Beasley et al.[5]

Random Selection of Cells and Measurement of Microvessel to Tumor Cell Distance

To compare the blood vessel to tumor cell distance for cells that did and did not express CA IX and for cells from areas of tumor with greater than or equal to and less than median staining, a selection of specimens was double stained for CA IX and CD34. Sections were initially stained with M75, and immunoreactivity was visualized with the Envision technique and DAB. Then the specimens were stained with anti-CD34, and immunoreactivity was visualized with alkaline phosphatase-antialkaline phosphatase and fuchsin (Table 1). To ensure random selection, specimens in the absent, low, moderate, and extensive mCA IX subgroups were allocated new identification numbers. A Microsoft Excel random number generator was then used to select pairs of specimens from each subgroup for double staining. Three areas from each specimen that encompassed tumor nests and blood vessels were photographed and printed onto A4 paper. Cells were randomly selected by placing a 10 × 6 square grid in the center of each photograph (Fig 3). Sixteen squares were randomly selected again using Excel (Fig 3). If a tumor cell was at the center of the selected square, the distance to the nearest blood vessel was measured using a pair of dividers (Fig 4). After a total of four measurements were taken from each photo, no further measurements were made. If tumor cells were found at the center of less than four squares, no further squares were chosen, and less than four measurements were accepted for that patient to avoid bias. The measurements were grouped first, as either from mCA IX–stained or unstained cells, and second, as either from areas of greater than or equal to mCA IX staining or from areas of less than median mCA IX staining, irrespective of whether the individual cell measured was stained.

Cell Culture, Lysis, Nuclear and Cytoplasmic Differential Extraction, and Immunoblotting

The cell line was A549 human pulmonary adenocarcinoma from American Type Culture Collection (ATCC, Manassas, VA). Cells were grown in DMEM (Sigma-Aldrich Company Ltd, Dorset, England), supplemented with 10% fetal calf serum (Gibco Life Technologies, Paisley, United Kingdom), L-glutamine (2 μM), penicillin (50 IU/mL), and streptomycin sulfate (50 μg/mL). Studies of inducible gene expression were performed on cells approaching confluence. Parallel incubations were performed on aliquots of cells in normoxia (humidified air with 5% CO_2) and hypoxia. Hypoxic conditions were generated in a NAPCO 7001 incubator (Precision Scientific, Winchester, VA) with 0.1% O_2, 5% CO_2, and the balance of N_2 unless otherwise specified. Experimental exposures were performed in normal growth medium for 16 hours.

Whole cell protein extracts were prepared from tissue culture cells prepared by 30 seconds of homogenization in denaturing conditions. For Western

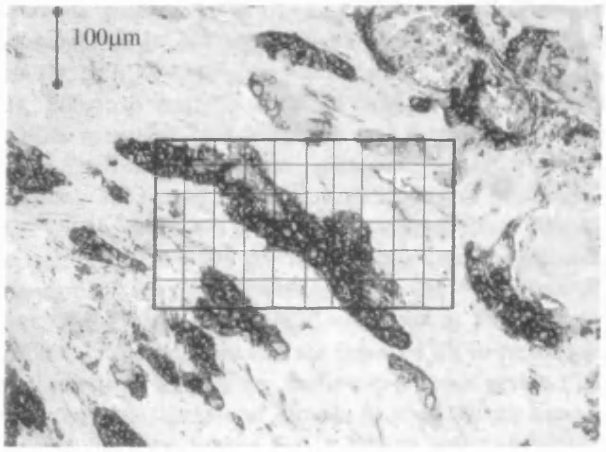

Fig 3. Non–small-cell lung cancer section stained with M75 (magnification ×100). Sixteen squares were randomly selected from the central grid and labeled. Measurements to the nearest vessel were then taken from the first four squares that had tumor cells at the center.

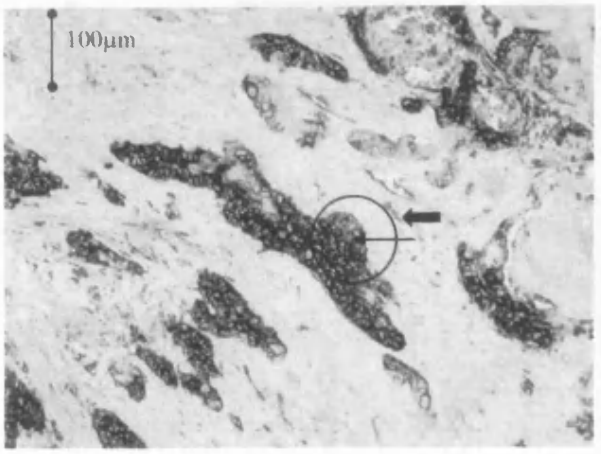

Fig 4. Non–small-cell lung cancer section stained with M75 magnification ×100. Measurements were taken from the selected tumor cells to the nearest blood vessel, labeled with a black arrow. A circle was drawn around the selected cell, the radius of which equaled the measured distance to ensure no vessels were closer.

immunoblotting, aliquots were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to Immobilon-P membranes (Millipore [U.K.] Ltd, Stonehouse, Gloucestershire, United Kingdom). CA IX was detected using M75 (1:50 for 16 hours at 4°C). Horseradish peroxidase–conjugated secondary antimouse antibody 1:2,000 in phosphate-buffered saline containing 5% fat-free skimmed milk (Marvel Premier Brand, Spalding, Lincolnshire, United Kingdom) and 0.1% Tween 20 (Dako) was applied at room temperature for 1 hour. Bound antibodies were detected using the chemiluminescent substrate ECL+Plus (Amersham Pharmacia Biotech, Amersham, United Kingdom).

Statistics

The SPSS software system (SPSS for Windows, version 9.0; SPSS Inc, Chicago, IL) was used to perform the statistical analysis as previously described.[21] For categorical analysis of mCA IX or cCA IX, high expression was defined as patients with greater than or equal to a median percentage of cells positive. pCA IX expression was either positive or negative, and increased stromal staining was defined as stromal score ≥ 2. The χ^2 test was used to analyze the associations between categorical variables. The statistical significance of trends between the increasing values of different categorical variables was analyzed using the linear-by-linear association test. Survival curves were plotted using the Kaplan-Meier method, and the statistical significance was assessed using the log-rank test. A Cox proportional hazards regression model was used to identify statistically significant differences in survival and estimate hazard ratios and 95% confidence intervals. The assumption of proportional hazards was assessed graphically by plotting log-minus-log of survival against time for each of the prognostic groups. Prognostic variables identified by univariate analysis with $P < .1$ were entered into a Cox multivariate analysis model. A forward, stepwise selection procedure was used, with variables added to the model according to a partial likelihood ratio test, using an entry criterion of $P < .05$.

RESULTS

CA IX Tumor Cell Expression

Tumor cell CA IX immunostaining was detected in 81.8% (142) of patients. Mixed patterns of cytoplasmic and membranous expression were seen in the majority of patients (Table 2) (Fig 5). The median percentage for both mCA IX and cCA IX staining patterns was 5% (range, 0% to 100%). mCA IX and cCA IX expression was observed in carcinoma-in-situ and entrapped bronchial epithelium within the tumor mass (Fig 6).

Table 2. CA IX Tumor Cell Expression

Tumor Cell Expression Pattern	No. of Patients (%)
Membranous	136 (77.7)
Cytoplasmic	129 (73.7)
Membranous and Cytoplasmic	123 (70.3)
Membranous only	13 (7.4)
Cytoplasmic only	6 (3.4)
Perinuclear	46 (26.3)
Nuclear	3 (1.7)
No expression	33 (18.86)

pCA IX expression was observed in occasional discrete tumor cells that always had mCA IX expression. Of pCA IX–positive patients (n = 46), 95.6% had high mCA IX expression and 66.7% were in the extensive mCA IX subgroup, demonstrating a close association between these two staining patterns. Three patients with pCA IX expression also expressed nuclear CA IX. Positive pCA IX expression also correlated with increased cCA IX expression ($P < .001$) but not with stromal CA IX expression.

There was significantly higher positive pCA IX and high mCA IX expression in the squamous carcinomas than in other NSCLC histologic subtypes ($P = .003$ and 0.008, respectively) (Table 3). pCA IX, mCA IX, and cCA IX expression were not associated with any other clinicopathologic parameters.

CA IX Stromal Cell Expression

CA IX stromal cell expression was observed in 111 patients. Increased stromal expression was associated with high mCA IX ($P = .003$) but not with either pCA IX or cCA IX. Stromal cell CA IX was significantly associated with advanced tumor stage ($P = .011$). Serial sections were stained with CD31 and CD34, markers for vascular endothelium, and vimentin and smooth muscle actin, which are markers for myofibroblasts[21-23] (see Table 1). A clear overlap was seen between CA IX, vimentin, and smooth muscle actin, indicating that the CA IX–positive stromal cells were myofibroblasts.

Associations With Tumor Necrosis

Patients with high mCA IX ($P = .005$), cCA IX ($P = .021$), and stromal ($P < .001$) expression were associated with extensive TN. pCA IX expression was not associated with TN ($P = .13$). Subgroup analysis dividing mCA IX expression into quartiles (negative, low, moderate, and extensive expression) demonstrated that there was a significant trend ($P = .036$) for increasing TN (tumor necrosis) with increasing expression of mCA IX. However, the χ^2 test for association found that when the mCA IX group was divided into quartiles, as opposed to binary groups, the significant association with TN was lost ($P = .054$). The loss of significance was because there were no significant differences between the extent of TN in the extensive group compared with the no- and low-expression groups (Table 4). For example, in 53% of patients (n = 25) in the extensive subgroup, there was limited TN. In 31% of patients (n = 12) in the no-expression, subgroup there was extensive TN.

Serial sections demonstrated that there was little or no CA IX expression adjacent to vascular endothelium expressing CD31

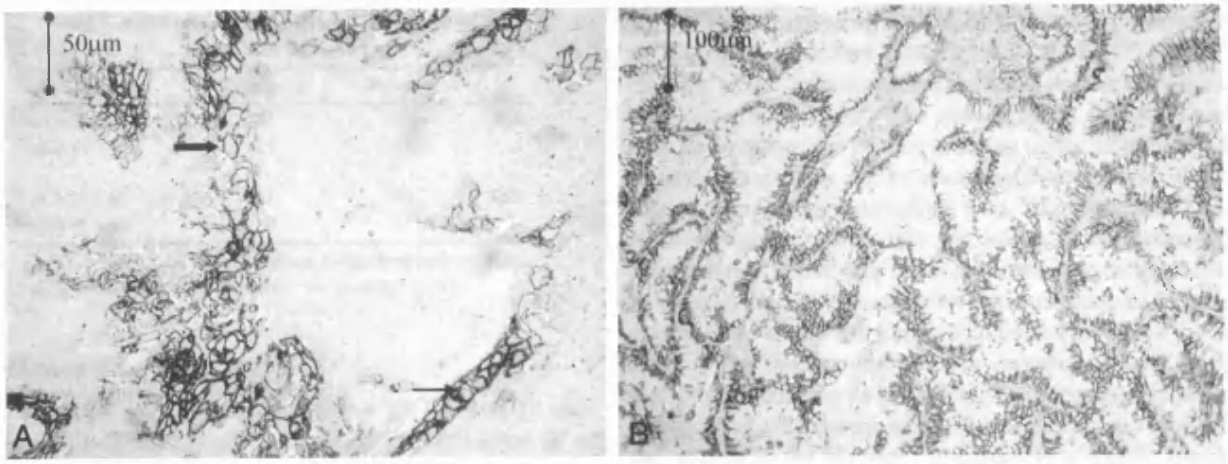

Fig 5. (A) Perinecrotic carbonic anhydrase (CA) IX cytoplasmic (thin arrow) and membranous (block arrow) expression in non–small-cell lung cancer (NSCLC) (magnification ×200). (B) Extensive membranous and cytoplasmic staining CA IX in NSCLC without tumor necrosis (magnification ×100).

and CD34, in contrast to the frequent perinecrotic expression (Fig 2).

Distance From Vascular Endothelium to the Start of CA IX Tumor Positivity

The median distance from the vascular endothelium to the tumor cells expressing CA IX was 90 μm (range, 48.7 to 142.5 μm; n = 18). The measured distances were an approximation because it was not possible to quantify tissue shrinkage during section-to-slide fixation. Thomlinson and Gray[24] published a formula that calculates the critical distance from a blood vessel to where TN commences. The calculations were based on examination of histologic specimens of squamous cell lung carcinoma. The formula makes assumptions of oxygen diffusion and oxygen consumption. Accepting that necrosis occurs when the $Po_2 = 0$, the formulas can be used to estimate the Po_2 a set distance from a blood vessel. The Po_2 90 μm from the blood vessel is calculated to be 5.77 mmHg or 0.76% O_2 (Fig 7).[21]

Mean Tumor Cell to Blood Vessel Distances for Randomly Selected Cells

There was a significant difference between the distances from tumor to blood vessel for mCA IX–positive tumor cells (mean = 117.1 μm, standard deviation [STD] 54.2 μm, n = 26) and mCA IX–negative tumor cells (mean distance = 43.2 μm, STD = 34.1 μm, n = 61) ($P < .001$). There was also a significant difference between the distances from tumor to blood vessel for tumor cells from areas of less than median staining (mean = 37.7 μm, STD = 21.6 μm, n = 47) and from areas of greater than or equal to median staining (mean = 103.6 μm, STD = 53.7 μm, n = 40) ($P < .001$), irrespective of whether the actual cell selected was mCA IX–positive.

A549 Cell Lines Express Nuclear CA IX

CA IX expression previously has been shown to be upregulated by increasing degrees of hypoxia in various cell lines.[10] Immunoblotting of whole cell extracts with M75 revealed a doublet of apparent molecular weights 54 and 58 kd in A549 cells. Differential extraction of nuclear (containing nuclear contents and membrane) and cytosolic (containing outer cell membrane and cytoplasmic proteins) contents demonstrated a nuclear enrichment of the 54-kd species, which, although enhanced by hypoxia, was present under normoxic conditions. The 58-kd species predominated in the cytosolic extract and was also enhanced by hypoxia (Fig 8).

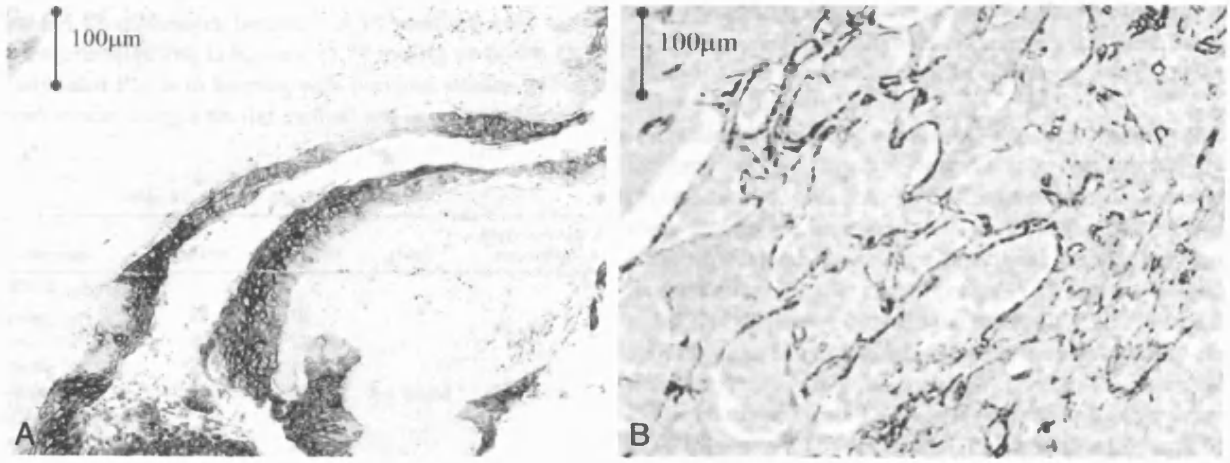

Fig 6. (A) Carcinoma-in-situ staining positive for carbonic anhydrase (CA) IX (magnification ×100). (B) Normal entrapped lung tissue within the tumor milieu staining positive for CA IX (magnification ×100).

Table 3. Associations With Squamous Histology for mCA IX and pCA IX Expression

CA IX Expression	Nonsquamous	Squamous	P
mCA IX −ve	43	48	.008
mCA IX +ve	23	61	
pCA IX −ve	57	72	.003
pCA IX +ve	9	37	

Abbreviations: mCA, membranous carbonic anhydrase (CA); pCA, perinuclear; −ve, negative; +ve, positive.

Correlations With a Poor Prognosis

Positive pCA IX (P = .036), extensive TN (P = .003), male sex (P = .034), increasing tumor-node-metastasis stage (P < .001), and high platelet count (P = .034) were univariate indicators of a poor prognosis. There was a trend toward a poor prognosis for high mCA IX patients (P = .11) and positive resection margins (P = .15) (Table 5). cCA IX and stromal expression were not associated with outcome (Fig 9 and Table 5). In multivariate analysis, increasing tumor stage (P < .001), high platelet count (P = .010), positive pCA IX expression (P = .004), male sex (P = .022), and extensive TN (P = .040) were independent poor prognostic factors (Table 6).

DISCUSSION

We have shown that pCA IX expression is an independent poor prognostic marker in NSCLC. pCA IX expression was only seen in cells with mCA IX staining, predominantly in patients with high mCA IX expression. Furthermore, high mCA IX expression is associated with extensive TN. Finally, mCA IX–positive cells are seen at an increased distance from the microvasculature compared with mCA IX–negative cells, in keeping with the postulate that hypoxia induces the enzyme. The frequency (81.8%) and predominant focal pattern of CA IX expression in this cohort is similar to that found in a previous series of 65 patients of NSCLC (80%).[13] The perinecrotic pattern of expression is consistent with findings from studies in other tumor types.[4-6]

The proposal by Wykoff[10] and Loncaster[11] that CA IX is a marker of tumor cell hypoxia is supported first by the associations between the extent of TN and mCA IX, cCA IX, and stromal CA IX expression. Second, CA IX positivity only starts when the estimated Po_2 is hypoxic (5.77 mmHg or 0.76% O_2). This estimated Po_2 is in keeping with previous studies in head and neck cancer using a similar method and is comparable to in vitro work demonstrating that CA IX is increasingly upregulated as the Po_2 is decreased from 2.5% to 0.1%.[5,10] An obvious criticism of the deduction that CA IX is a marker of hypoxia on the basis of the strength of the estimated Po_2, is that the method used incorporates bias by only selecting tumor sections that have extensive expression and by excluding blood vessels that have been cut along their longitudinal axis. To counter this bias, we randomly selected tumor cells and measured the distance to the nearest blood vessel. The mean distances for positive cells and for cells from areas of tumor with high positivity found with this method were similar but higher than those found with the initial method. This discrepancy would be expected because the initial method measured the distance from the nearest tumor cell to the blood vessel, whereas the second method selected a random cell and measured the distance. Importantly, a highly significant difference was found between tumor cells that expressed CA IX and those that did not, strengthening the proposal that CA IX is a hypoxic marker. We have also demonstrated that hypoxia enhanced CA IX expression in the cytosolic and nuclear extract of A549 cells.

Table 4. mCA IX Associations With TN

Subgroups	Focal TN	Extensive TN	χ^2 Test	Linear-by-Linear Association
Four mCA IX subgroups				
Negative	27	12		
Low	26	12		
Moderate	23	28		
Extensive	25	22	P = 0.054	P = 0.036
Two mCA IX subgroups				
< Median	53	24		
≥ Median	48	40	P = 0.005	

Abbreviations; mCA, membranous carbonic anhydrase; TN, tumor necrosis.

The presence of a range of distances between microvessels and CA IX–positive tumor cells, as opposed to a set distance, has two explanations. First, there may be differences in tissue oxygen use throughout the tumor. Second, blood flow through often-fragile tumor microvessels may be intermittent, which would cause variations in the degree of hypoxia at varying distances from the microvessels.

Squamous carcinomas are known to be more necrotic than other NSCLC histologic subtypes, suggesting that they are more hypoxic.[25] In keeping with this observation, and the correlation between TN and mCA IX expression in this study, a higher expression of mCA IX was found in squamous tumors. The association between pCA IX and squamous carcinomas is not so readily explained; pCA IX was not associated with TN. However, pCA IX was only observed in mCA IX–positive cells, which suggests that hypoxia is in part responsible for pCA IX expression. Alternatively, a secondary process may be responsible for pCA IX expression that is more active in squamous carcinomas. Entrapped nonmalignant bronchial epithelium and, in particular, carcinoma-in-situ, often expressed mCA IX, which reflects the hypoxic nature of these tissues in contrast to adjacent normal lung tissue that never stained for CA IX. The lack of CA IX expression around microvessels suggests that these tissues are relatively well oxygenated and, by inference, that the microvessels are functionally active.

Certain observations were made that were inconsistent with the proposal that hypoxia was the sole regulator of CA IX expression. On occasion, CA IX expression was observed in areas that would be expected to be well oxygenated, such as adjacent to blood vessels, and in patients with little or no TN. Furthermore, subgroup analysis of mCA IX expression found no significant difference between the extent of TN in the extensive and moderate subgroups and between the low- and no-expression subgroups. Finally, as previously stated, pCA IX expression did not correlate with TN even though all cells that expressed pCA IX coexpressed mCA IX. The transcription factor HIF-1 regulates CA IX; hence, aberrant regulation of HIF-1 would be expected to increase expression of CA IX. Disregulation of HIF-1

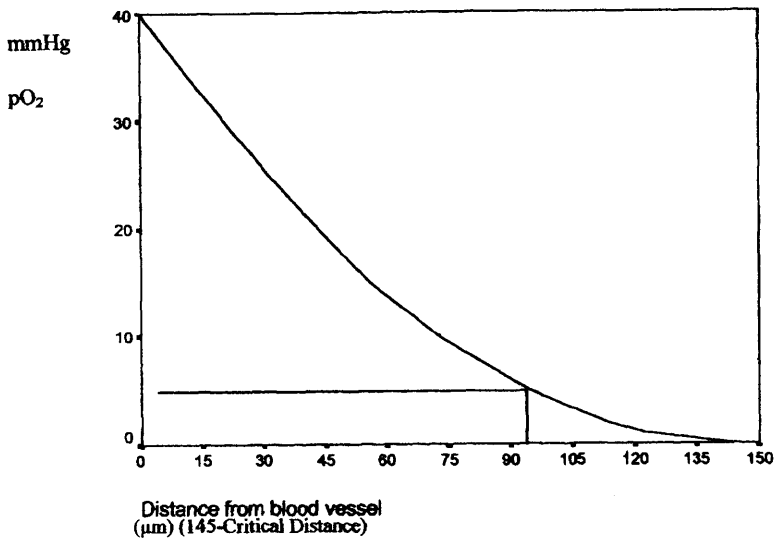

Fig 7. A graphic representation of Thomlinson and Gray's equation estimating the oxygen pressure a set distance from a blood vessel.

$$\text{Critical Distance (cm)} = \sqrt{\frac{4 \times O_2 \text{ diffusion coefficient (DC)} \times pO_2(\text{mmHg})}{O_2 \text{ consumed/ ml/ second (M)}}}$$

$$DC/M = 1.31 \times 10^{-6}$$

could be caused by mutation of the von Hippel-Lindau (VHL) tumor suppressor gene or activation of the epidermal growth factor receptor (EGFR). Mutated VHL prevents appropriate normoxic degradation of HIF-1 and, as such, CA IX is heavily expressed in mutated VHL-related tumors, independently of the extent of TN.[10] VHL gene mutations have previously been demonstrated in a proportion of cell lines derived from thoracic malignancies.[26] VHL mutations may be present in the subgroup of patients with extensive

Table 5. Prognostic Significance of CA IX Staining and Clinicopathologic Parameters

Prognostic Factor		n	Hazard Ratio	95% CI	P
Age, years	< Median (66)	87	1.0		.53
	≥ Median (66)	88	1.12	0.79 to 1.57	
Sex	Female	50	1.0		.034
	Male	125	1.54	1.03 to 2.29	
Histology	Adenocarcinoma	48	1.0		.29
	Large	14	0.85	0.39 to 1.85	
	Squamous	109	1.33	0.89 to 1.99	
	Other	4	1.8	0.65 to 5.19	
Grade	Well/mod diff	91	1.0		.62
	Poor diff	84	1.09	0.77 to 1.53	
TNM stage	1	84	1.0		< .001
	2	51	1.83	1.22 to 2.73	
	3	40	2.60	1.69 to 3.98	
Positive resection margins	Negative	160	1.0		
	Positive	15	1.50	0.86 to 2.61	.150
Platelet count	< Median (314)	84	1.0		.034
	≥ Median (314)	85	1.46	1.03 to 2.07	
Tumor necrosis	Focal	101	1.0		.003
	Extensive	74	1.67	1.19 to 2.35	
CA IX perinuclear	Positive	46	1.0		.036
	Negative	129	1.50	1.03 to 2.19	
CA IX membranous	< Median (5%)	91	1.0		.11
	≥ Median (5%)	84	1.32	0.94 to 1.85	
CA IX cytoplasmic	< Median (5%)	102	1.0		.18
	≥ Median (5%)	73	1.25	0.89 to 1.77	
CA IX stromal	0-2	108	1.0		.91
	2-3	67	1.02	0.72 to 1.45	

Abbreviations: CI, confidence interval; TNM, tumor-node-metastasis; CA, carbonic anhydrase.

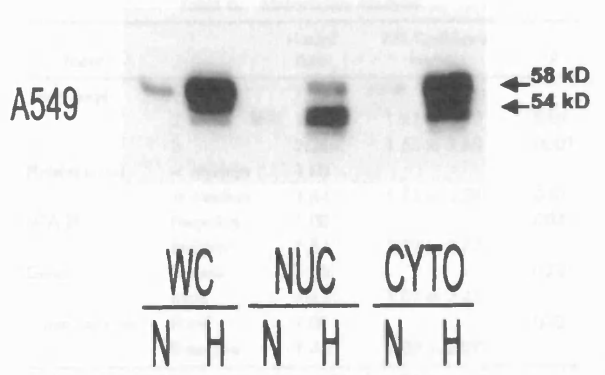

Fig 8. Western immunoblot of nuclear and cytosolic carbonic anhydrase IX extract under normoxic and hypoxic conditions demonstrating the 54 and 58 kd doublet.

mCA IX expression and focal TN. With regard to EGFR signaling, in vitro studies show that activation of EGFR stimulates the phosphatidylinositol 3-kinase pathway, which in turn can upregulate HIF-1 alpha independently of hypoxia.[27] Recent work has demonstrated that EGFR activation is able to augment hypoxic induction of another HIF-1 alpha transcription target, vascular endothelial growth factor (VEGF), via the phosphatidylinositol 3-kinase pathway.[28] This suggests that EGFR activation may also augment induction of CA IX. The pCA IX group, which consists of patients with predominantly extensive mCA IX, may represent such processes acting in concert with tumor hypoxia.

Conversely, TN was commonly observed in the absence of CA IX staining. This may represent infarction of nonhypoxic tissue caused by an acute ischemic event. Ausprunk et al[29] showed that tumor microvasculature is extremely fragile and can easily be damaged. Under such circumstances an acute thrombus may occur, resulting in TN.

The structures responsible for pCA IX positivity are unknown. However, this occasional pattern of staining may represent CA IX protein in the endoplasmic reticulum during periods of increased synthesis.[30] Supporting this is the observation that pCA IX was predominantly found in patients with high mCA IX expression.

Immunoblotting of whole cell extracts with M75 has previously been reported to demonstrate a doublet of apparent molecular weights 54 and 58 kd in cultured HeLa cells grown in dense cultures and tumorigenic HeLa cross fibroblast hybrids. Using immunoelectron microscopy, the CA IX staining was localized to the surface microvilli and the nucleus, particularly in the nucleoli in the HeLa cells.[18,31] The presence of nuclear CA IX in three patients in our series shows that this is a rare but reproducible feature in NSCLC. In relation to this observation, a nuclear protein with CA enzymatic activity has been identified in human and rat testis and rat lymphocytes. This protein appeared to be identical to the transcription factor NonO/p54^{nrb} on amino acid sequencing. NonO/p54^{nrb} lacks the structural elements heretofore considered essential for zinc binding, which is essential for CA activity and as such has been classified as a nonclassical CA. The role of nuclear CA IX is as yet undetermined, but the observation of a known transcription factor with CA activity suggests that CA IX may also act as a transcription factor.[32]

In NSCLC, breast, and cervical cancer CA IX expression is detected in premalignant lesions, which suggests that hypoxia and CA IX are involved at an early stage in tumorigenesis.[12] Our observation of CA IX expression in carcinoma-in-situ supports this hypothesis.

The finding that positive resection margins approached significance as a univariate indicator of a poor prognosis is possibly the result of the small number of patients. Of note is the association between stage IIIA (N2) disease and the presence of

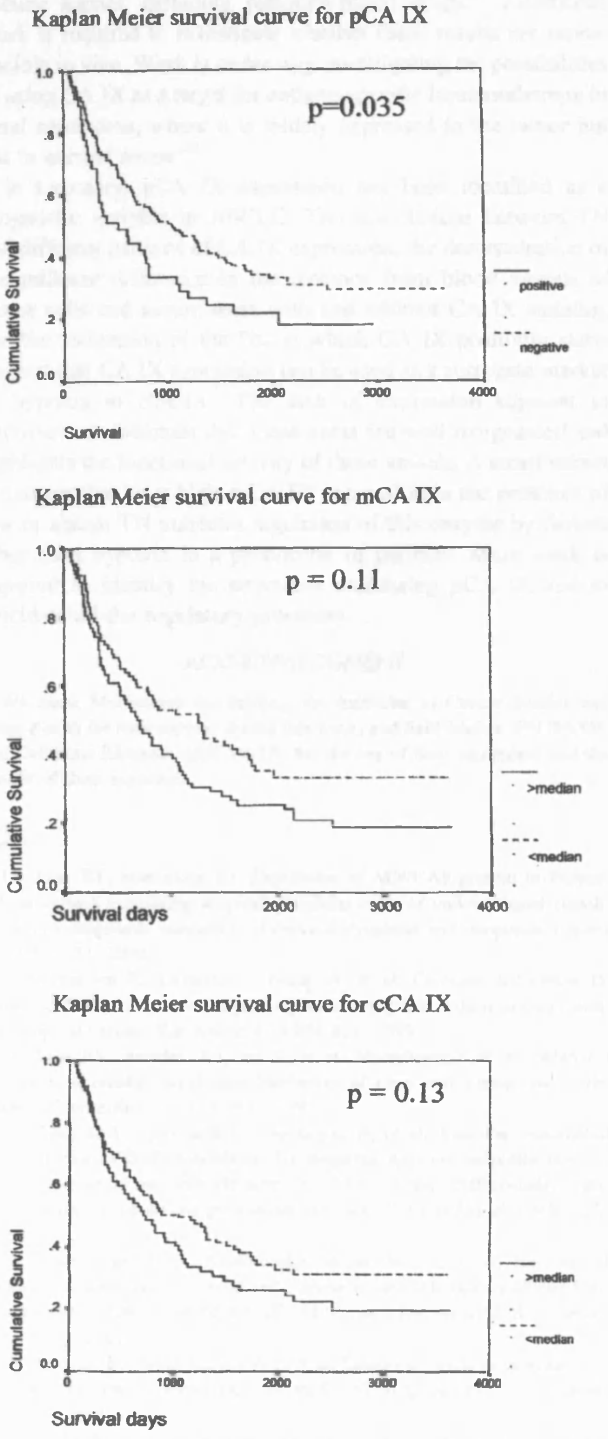

Fig 9. Kaplan-Meier survival curves for perinuclear carbonic anhydrase (CA) IX, membranous CA IX, and cytoplasmic CA IX expression.

Table 6. Multivariate Analysis

Factor		Hazard Ratio	95% Confidence Interval	P
TNM stage	1	1.00		
	2	1.57	1.02 to 2.40	.035
	3	2.38	1.53 to 3.68	.0001
Platelet count	< Median	1.00		
	≥ Median	1.64	1.13 to 2.39	.010
pCA IX	Negative	1.00		.004
	Positive	1.84	1.22 to 2.77	
Gender	Female	1.00		.022
	Male	1.63	1.07 to 2.47	
Tumor necrosis	Focal	1.00		.040
	Extensive	1.45	1.02 to 2.07	

Abbreviations: TNM, tumor-node-metastasis; pCA IX, perinuclear carbonic anhydrase IX.

positive resection margins. Previous studies of larger populations report that the prognosis for positive resection margins is similar to that of stage IIIA (N2) disease. However, studies differ about whether the poor prognosis conferred by positive margins is independent of that conferred by advanced stage of disease.[33-36]

CA IX may be a therapeutic target for cancer. Inhibition of CA isoenzymes with acetazolamide or sulfonamides results in either reduced tumor invasiveness or blocked tumor growth, respectively.[37,38] Furthermore, CA isoenzyme antagonism has been observed to augment the cytotoxic effects of various chemotherapeutic agents, including platinum-based drugs.[39] Additional work is required to investigate whether these results are reproducible in vivo. Work is under way investigating the possibilities of using CA IX as a target for antigen-specific immunotherapy in renal carcinoma, where it is widely expressed in the tumor but not in normal tissue.[40]

In summary, pCA IX expression has been identified as a prognostic variable in NSCLC. The associations between TN and different patterns of CA IX expression, the demonstration of a significant difference in the distance from blood vessels of tumor cells and tumor areas with and without CA IX staining, and the estimation of the Po_2 at which CA IX positivity starts suggest that CA IX expression can be used as a surrogate marker of hypoxia in NSCLC. The lack of expression adjacent to microvessels indicates that these areas are well oxygenated and highlights the functional activity of these vessels. A small subset of tumors that have high mCA IX expression in the presence of low or absent TN indicates regulation of this enzyme by factors other than hypoxia in a proportion of patients. More work is required to identify the structures expressing pCA IX and to elucidate all the regulatory processes.

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HYPOXIA INDUCIBLE FACTOR-1α IN NON-SMALL CELL LUNG CANCER: RELATION TO GROWTH FACTOR, PROTEASE AND APOPTOSIS PATHWAYS

Daniel E. B. Swinson[4], J. Louise Jones[2], Giles Cox[1], Donna Richardson[1], Adrian L. Harris[3] and Kenneth J. O'Byrne[1]

University Departments of Oncology[1] and Pathology[2], University Hospitals of Leicester NHS Trust, Leicester, LE1 5WW, UK.

Imperial Cancer Research Fund, Molecular Oncology Unit, Institute of Medicine, John Radcliffe Hospital, Oxford OX3 9DS, UK[3].

Cancer Centre, Queen Elizabeth Hospital, Edgbaston, Birmingham, B, UK[4].

Corresponding Author: Dr Daniel Swinson

Tel: +44-121-627-2444 or +44-7958620077 Fax: +44-121-627-2496

E-mail address: daniel.swinson@uhb.nhs.uk

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Key words: Non-small cell lung cancer, HIF-1α, Hypoxia, CA IX, EGFR, MMP-9

Abbreviations used: HIF: hypoxia inducible factor; EGFR: epidermal growth factor; MMP: matrix metalloproteinase; NSCLC: Non-small cell lung cancer; CA: carbonic anhydrase; TN: tumour necrosis; pVHL: Von Hippel Landau protein; ODDD: Oxygen dependent degradation domain; MAPK: mitogen activated protein kinase: PI-3K: phosphoinositidyl-3 kinase; VEGF: vascular endothelial growth factor; TGF: transforming growth factor; MVC: microvessel count; CSA: Catalysed signal amplification; TBS: tris buffered saline; SCCHN: squamous cell carcinoma of the head and neck; NF: nuclear factor

ABSTRACT

Hypoxia inducible factor (HIF)-1α is the regulatory subunit of HIF-1 that is stabilised under hypoxic conditions. Under different circumstances HIF-1α may promote both tumourigenesis and apoptosis. There is conflicting data on the importance of HIF-1α as a prognostic factor. This study evaluated HIF-1α expression in 172 consecutive patients with stage I-IIIA Non-small cell lung cancer (NSCLC) using standard immunohistochemical techniques. The extent of HIF-1α nuclear immunostaining was determined using light microscopy and the results were analyzed using the median (5%) as a low cut point and 60% as a high positive cut point. Using the low cut point, positive associations were found with epidermal growth factor receptor (EGFR) ($p=0.01$), matrix metalloproteinase (MMP)-9 ($p=0.003$), membranous ($p<0.001$) and perinuclear ($p=0.004$) carbonic anhydrase (CA) IX, p53 ($p=0.008$), T-stage ($p=0.042$), tumour necrosis (TN) ($p<0.001$) and squamous histology ($p<0.001$). No significant association was found with Bcl-2 or either N- or overall TMN stage or prognosis. When the high positive cut point was used HIF-1α was associated with a poor prognosis ($p=0.034$). In conclusion the associations with EGFR, MMP-9, p53 and CA IX suggest that these factors may either regulate or be regulated by HIF-1α. The association with TN and squamous type histology, which is relatively more necrotic than other NSCLC types, reflects the role of hypoxia in the regulation of HIF-1α. The prognostic data may reflect a change in the behaviour of HIF-1α in increasingly hypoxic environments.

INTRODUCTION

Tissue hypoxia is a fundamental characteristic of solid tumours and promotes biological processes involved in tumour progression (Harris, 2002). High levels of tumour hypoxia have been reported to confer a poor prognosis in cancers of the uterine cervix, head and neck and soft tissue sarcomas (Hockel *et al.*, 1991; Nordsmark *et al.*, 1996; Nordsmark *et al.*, 1996). This effect was initially thought to be restricted to hypoxia's influence on response to radiotherapy. However, studies in cervical cancer have found the prognostic impact to be independent of therapeutic mode (Hockel *et al.*, 1996). Moreover secondary cancers are more hypoxic than respective primaries of similar sizes (Hockel *et al.*, 1998).

Semenza et al. identified the hypoxia inducible factor (HIF)-1 transcription factor in 1992. HIF-1 is a heterodimer consisting of 2 subunits HIF-1α and HIF-1β (otherwise known as the aryl hydrocarbon receptor nuclear translocator) that is stabilised by hypoxia (Semenza and Wang, 1992).

The expression of these subunits differ, HIF-1β is constitutively expressed unlike HIF-1α that is rapidly degraded under normoxic conditions. In the presence of oxygen (O_2) HIF-1α is ubiquitinated after binding to the tumour suppressor von Hippel Lindau protein (pVHL), a component of the multi-protein ubiquitin E3 ligase complex. Subsequently HIF-1α is taken up and degraded by proteosomes (Huang *et al.*, 1998; Salceda and Caro, 1997). Hypoxia inhibits hydroxylation of prolyl residues 402 and 564 in the oxygen dependent degradation domain (ODDD) that prevent binding of the pVHL. Similar hypoxia dependent inhibition of hydroxylation of asparagines residues within the C-terminal activation domain increases HIF-1 transcriptional activity (Ivan *et al.*, 2001;

Jaakkola *et al.*, 2001; Lando *et al.*, 2002; Masson, 2001). Oxygen dependent degradation of HIF-1α is inhibited by the v-Src and Ras V12 oncogenes that interfere with prolyl hydroxylation (Chan *et al.*, 2002).

Growth factors, cytokines and oncogenes stimulating the p42/p44 mitogen associated protein kinase (MAPK) and/ or phosphoinositidyl-3 kinase (PI-3K) pathways may increase HIF-1α activity by either phosphorylating or increasing the expression of HIF-1α independently of O_2. The importance of these signalling pathways to the regulation of HIF-1α has however been inconsistent in different cell lines (Alvarez-Tejado *et al.*, 2002; Arsham *et al.*, 2002; Semenza, 2000).

HIF-1 binds to a conserved sequence (5'-CGTG-3') known as the hypoxic response element in the promoter region of its target genes. Erythropoietin was the first target to be identified since when a myriad of different targets have been defined (Semenza and Wang, 1992). These target genes are involved in a wide range of processes that promote cellular survival. Such processes include angiogenesis, blood vessel vasodilatation and erythropoiesis, anaerobic metabolism, buffering of the intracellular compartment and induction of growth factors (Harris, 2002; Semenza, 2000). HIF-1α activity *in vivo* has been found to promote tumour growth in the majority of studies and resistance to various chemotherapy agents including platinum compounds (Jiang *et al.*, 1997; Ryan *et al.*, 1998; Ryan *et al.*, 2000; Unruh *et al.*, 2003). A study by Carmeliet *et al.* has reported results to the contrary (Carmeliet *et al.*, 1998). The phosphorylation status of HIF-1α may determine whether it acts to protect or check tumour cell survival as dephosphorylated HIF-1α stabilises p53 inducing apoptosis where as phosphorylated HIF-1α binds to HIF-1β to form the HIF-1 complex (Suzuki *et al.*, 2001).

HIF-1α is widely expressed in solid tumours and has been found to be associated with both an improved and a worse prognosis in different tumour types, the results of which are summarized in table 1. Carbonic anhydrase (CA) IX and glucose transporter-1 are transcriptional targets of HIF-1α and along with HIF-1α have been identified as novel markers of hypoxia in different tumour types (Airley *et al.*, 2001; Beasley *et al.*, 2001; Loncaster *et al.*, 2001; Swinson *et al.*, 2003; Wykoff *et al.*, 2000).

A previous study in Non-small cell lung cancer (NSCLC) found HIF-1α was associated with expression of vascular endothelial growth factor (VEGF), platelet derived endothelial cell growth factor and basic-fibroblastic growth factor but not significantly with prognosis (Giatromanolaki *et al.*, 2001).

This study was designed to further examine the role of HIF-1α in NSCLC and to compare its expression to other biological factors that may either regulate or be regulated by this transcription factor.

MATERIALS AND METHODS

Study Population.

The study population consisted of 204 consecutive cases of resected stage I-IIIA NSCLC from patients who had undergone surgery with curative intent. Of these, 24 were excluded due to death within 60 days of surgery to reduce the confounding variable of peri-operative mortality and 8 further cases were excluded due to lack of available tissue. Of the 172 cases stained, 120 were male and 52 were female. There were 107 squamous carcinoma, 49 adenocarcinoma, and 12 large cell carcinoma and in 4 patients the histological subtype could not be defined. The median age at surgery was 65.9 years

(range 33.8-79.1). Positive resection margins were found in 13 cases. One case received adjuvant chemotherapy and 14 cases were treated with adjuvant radiotherapy. The final staging was based on the findings at surgery and the histopathology report. Hospital notes of the patients were reviewed and if necessary the local cancer registry or patient's General Practioner were contacted to complete case follow up. From this data it was established that 130 patients had died at the time of analysis and of these deaths 16 were not cancer related and in 4 the cause of death was unknown. Survival data was based on a median follow up of 90.6 months, range of 61.7-130 months for patients alive.

Materials

The specimens studied were routinely processed, formalin-fixed and embedded in paraffin blocks. Only blocks containing the advancing edge of the primary tumour were evaluated. Tissue sections of 4μ thickness were cut onto glass slides that were previously treated with 2% 3-aminopropylethoxysilane (in methanol) and dried overnight at 37°C to assist with section to slide adhesion.

Immunohistochemistry

The expression of HIF-1α was analysed using the anti-HIF-1α monoclonal antibody (clone Mab H1α67, NB 100-105; Novus Biologicals, Liddleton, CO; (Zhong *et al.*, 1999). According to manufacturers specifications this antibody recognises bands at M_r 120 000 in Western blot, representing HIF-1α in activated cells and has also been used to immunoprecipitat human HIF-1α. Visualisation of immunopositivity was performed using the Catalysed signal amplification kit (CSA) (Dako, UK). Sections were submerged in Dako antigen retrieval solution at 97°C for 45 minutes. Endogenous peroxidase was blocked by immersion of sections in 3% hydrogen peroxide for 5 minutes.

Background staining was blocked by incubation with a protein blocking solution (CSA kit). Primary antibody was applied at a dilution of 1:1000 in Tris buffered saline (300mM NaCl, 50mM Tris-HCl, 0.1% Tween 20) pH 7.6 (TBS) and incubated overnight at 4°C. Streptavidin-biotin-peroxidase complex (CSA kit) was applied for 15 minutes followed by an amplification reagent (CSA kit) for 15 minutes. Streptavidin-peroxidase (CSA kit) was then applied for 15 minutes and finally diaminobenzidine substrate for 5 minutes. Between steps sections were washed by 5-minute submersions in TBS. Sections were dehydrated by reverse passage through alcohols, counter stained with haematoxylin and mounted with xylene-based mountant.

Clear cell renal carcinoma specimens that were known to express HIF-1α were used as positive controls. For the negative controls the primary antibody was omitted and sections were incubated with TBS alone.

The expression of tumour cell HIF-1α was analysed by semi-quantitatively assessing the percentage of tumour cells expressing nuclear HIF-1α. Intensity of staining was not included in the scoring system as there was little variation between cases. When an increase in intensity was observed it was associated with increased background staining. The extent of tumour necrosis (TN) was evaluated and defined as either limited or extensive (Swinson *et al.*, 2002).

Previous immunohistochemistry

The expression of epidermal growth factor receptor (EGFR), matrix metalloproteinase (MMP)-9, CA IX, p53 and microvessel counts (MVC) have previously been analysed in this series (table 2) (Cox *et al.*, 2000; Cox *et al.*, 2000; Cox *et al.*, 2000; Swinson *et al.*, 2003).

Statistics

The SPSS software system (SPSS for Windows Version 9.0, www.SPSS.com) was used to perform the statistical analysis. For statistical analysis the series was dichotomised twice; $\geq$ median (5%) was used as a low cut point to define negative or positive HIF-1α staining and $\geq$ 60% was used as a high positive cut point to define high positive or low HIF-1α staining. The χ^2-test was used to analyse the associations between categorical variables. This test was used as opposed to the Fisher's exact test as there were no subgroup sizes less than 5. Survival curves were plotted using the Kaplan-Meier method and the statistical significance was assessed using the log-rank test. A Cox proportional hazards regression model was used to identify statistically significant differences in survival and estimate hazard ratios and 95% Confidence intervals. The assumption of proportional hazards was assessed graphically by plotting log-minus-log of survival against time for each of the prognostic groups. Prognostic variables identified by univariate analysis with $p<0.1$, were entered into a Cox multivariate analysis model. A forward, stepwise selection procedure was used, with variables being added to the model according to a partial likelihood ratio test, using an entry criterion of $p<0.05$. The Excel random number generator was used to select random cases for re-interpretation.

RESULTS

Tumour expression of HIF-1α

Nuclear HIF-1α expression was observed in 101 patients (55.8%). The median percentage of positive tumour cells was 5%, range 0-100%. Positive (≥ 5%) and high positive (≥60%) expression was observed in 80 and 22 patients, respectively. The staining was predominantly perinecrotic (figure 1). No expression was observed in either tumour stroma or adjacent normal tissue.

Associations with clinico-pathological and biological variables

Positive HIF-1α expression was associated with the following clinico-pathological and biological variables: increasing T-stage (p=0.042), squamous histology (p<0.001), extensive TN (p<0.001), moderate MVC (p=0.007) and EGFR (p=0.01), perinuclear (p) (p=0.004) and membranous (m) (p<0.001) CA IX, MMP-9 (p=0.003) and p53 (p=0.008). No association was found with N- or overall stage, gender, positive tumour margins or Bcl-2 (table 3).

Survival analysis

High positive HIF-1α expression was associated with a poor prognosis (median survival and standard errors high positive HIF-1α expression: 72 +/- 14.6; low HIF-1 expression: 59+/- 26.1 months, p=0.034) (figure 2). Positive HIF-1α expression was not associated with prognosis (p=0.52). When the percentage of staining was used as a continuous variable, HIF-1α was non-significantly associated with a poor prognosis (p=0.07). Other studies have divided series of patients into 3 groups with high, medium and low staining. When the series was divided into 3 groups high positive (≥60%),

positive (≥5%-<60%) and negative (<5%), no significant association was found with prognosis (p=0.1).

Prognostic data from previous studies was revised with updated survival data. On univariate analysis using the log rank test, biological variables pCA IX expression (p=0.03) and MMP-9 expression (p=0.002) and clinico-pathological variables TN (p=0.005), MVC tertiles (p=0.013), stage (p<0.001), gender and platelet count remained associated with a poor prognosis (Cox *et al.*, 2000; Cox *et al.*, 2000; Swinson *et al.*, 2003). Bcl-2 (p=0.58) had lost its previous association with prognosis (Cox *et al.*, 2000). P53 (p=0.57), mCA IX (p=0.11), EGFR (p=0.72) expression and the clinico-pathological variables positive tumour margins, histological subtype and grade were still not associated with prognosis (table 4) (Cox *et al.*, 2000; Cox *et al.*, 2000; Cox *et al.*, 2000).

High positive HIF-1α (p=0.006) was an independent prognostic variable when the clinico-pathological variables stage, gender and platelet count were analysed in multivariate analysis (table 5). When novel clinico-pathological variables MVC and TN and biological variables pCA IX and MMP-9 were included in multivariate analysis high positive HIF-1α was not an independent variable (table 6).

Reproducibility of interpretation

Two independent observers scored 20 randomly selected slides as having high positive, positive or negative expression. Agreement was achieved in each case.

DISCUSSION

Here we show that HIF-1α is commonly expressed in NSCLC and is associated with a number of biological factors that are involved in the pathogenesis of NSCLC. When a high positive cut point is used, HIF-1α expression is associated with a poor prognosis and this was found to be independent of standard clinicopathological factors. When novel biological and clinicopathological factors were included in multivariate analysis the prognostic relevance of high positive HIF-1α expression was washed out.

The prognostic significance of HIF-1α expression has now been evaluated in a number of solid tumours. Increased HIF-1α expression has been reported to be associated with a poor prognosis in lymph node positive breast cancer, non-metastatic oropharangeal cancer and oligodendroma (Aebersold *et al.*, 2001; Birner *et al.*, 2001; Schindl *et al.*, 2002) (table 1). Conflicting results have been reported in cervical carcinoma and in squamous cell carcinoma of the head and neck (SCCHN). In early stage cervical carcinoma treated with surgery HIF-1α expression was found to be strongly associated with a poor prognosis. In locally advanced cervical carcinoma treated with radiotherapy, HIF-1α was not prognostic. This was surprising as HIF-1α expression in the later study did correlate with tumour hypoxia (Birner *et al.*, 2000; Haugland *et al.*, 2002). In early stage SCCHN treated with surgery, the presence of HIF-1α expression conferred a good prognosis as opposed to locally advanced disease, treated with chemo-radiotherapy where an association with a poor prognosis was found (Beasley *et al.*, 2002; Koukourakis *et al.*, 2002).

A possible explanation for the variability in the results seen may be due to the cut points used to define cases as over expressing HIF-1α. In the studies that did not show

HIF-1α to be associated with a poor prognosis, the cut-points used were relatively low, being ≤ 5% of HIF-1α positive cells. In contrast, studies reporting HIF-1α to be associated with a poor prognosis have used higher cut points. An exception is a study of epithelial ovarian carcinomas where the series was divided into quartiles. HIF-1α expression correlated with the apoptotic rate especially in the low malignant tumours and no association was found with prognosis. However in cases with strong HIF-1α expression *i.e.* the upper quartile and with p53 co-expression, an association with a poor prognosis was observed (Birner *et al.*, 2001). Taking the results of these separate studies and ours as a whole they suggest extensive HIF-1α expression may reflect extremes in tumour hypoxia. This may need to be present in order for HIF-1α to have a pro-tumourigenic effect. This postulate is also in agreement with studies demonstrating that HIF-1α has both pro- and anti-tumour properties (Carmeliet *et al.*, 1998; Maxwell *et al.*, 1997; Suzuki *et al.*, 2001).

High HIF-1α expression may reflect chronic as opposed to acute hypoxia and dictate prognosis in the longer rather than the short term. A prognostic impact would therefore become more apparent in a population with longer-term survival such as those treated with surgery for early disease rather than those with more advanced disease treated with radiotherapy. Furthermore, although acute hypoxia is important in dictating the response to radiotherapy it may be less important than chronic hypoxia in dictating the long term response to surgery and chemotherapy (Airley *et al.*, 2003). This may explain the disparity between the 2 studies in cervical cancer (table 1) (Birner *et al.*, 2000; Haugland *et al.*, 2002). A similar observation has been made for the prognostic impact of the extent of TN, another surrogate marker of hypoxia in NSCLC, where although

extensive TN predicts outcome in early stage disease no such effect is seen in locally advanced disease (Swinson *et al.*, 2002). Against this hypothesis is the observation that the Kaplan Meier curves in our study separate relatively early. Furthermore a number of other studies have included patients with locally advanced disease in different cancer types and reported an association between HIF-1α expression and prognosis (table 1).

The associations between HIF-1α, CA IX, TN and squamous histology are consistent with the recognised mechanisms that regulate and are regulated by HIF-1α. CA IX is regulated by HIF-1α. TN occurs as a result of chronic hypoxia and squamous carcinomas are known to be more necrotic than other NSCLC histological subtypes (Dail and Hammar, 1998; Thomlinson and Gray, 1955; Wykoff, 2000). Both TN and CA IX have been associated with a poor prognosis in NSCLC suggesting that in a subpopulation of cases, tumour cells are able to escape the apoptotic properties of HIF-1α (Swinson *et al.*, 2002; Swinson *et al.*, 2003).

The associations of HIF-1α with EGFR and MMP-9 have not previously been reported in immunohistochemical studies. Upon ligand binding EGFR may activate either or both the PI-3K and the p42/ p44 MAPK pathways. Activation of the p42/ p44 MAPK pathway may stimulate phosphorylation of HIF-1α or alternatively upregulate HIF-1α expression (Fukuda *et al.*, 2002; Semenza, 2000). In prostate cell lines EGF stimulation of the PI-3K pathway increased HIF-1α expression and transcriptional activity (Jiang *et al.*, 2001; Semenza, 2000). Other studies in tumour cell lines have also reported that stimulation of the PI-3K or p42/ p44 MAPK pathways induces HIF-1α expression and transcriptional activity (Blancher *et al.*, 2001; Chan *et al.*, 2002; Jiang *et al.*, 2001; Laughner *et al.*, 2001; Sandau *et al.*, 2001; Stiehl *et al.*, 2002). This is not a

consistent finding as two recent studies failed to demonstrate such a result (Alvarez-Tejado *et al.*, 2002; Arsham *et al.*, 2002). Nonetheless, the correlation between EGFR and HIF-1α expression seen in our study suggests that EGFR may influence the level of HIF-1α expression in NSCLC acting via either or both the PI-3K and p42/ p44 MAPK pathways. Another explanation may be that increased EGFR signalling induces increased tumour cell proliferation sufficient to generate tumour hypoxia hence increasing HIF-1α expression.

MMP-9 is an endopeptidase that facilitates tumour invasion and angiogenesis through breakdown of the extracellular matrix. MMP-9 mRNA levels are regulated by a number of transcription factors including nuclear factor (NF)-kappa B and activator protein-1, which can be induced by the p42/ p44 MAPK and PI-3K pathways in response to cytokines and growth factors (Bond *et al.*, 1998; Eberhardt *et al.*, 2000). To date HIF-1α has not been implicated in the regulation of MMP-9 although some *in vitro* work has found the enzyme to be induced by hypoxia (Canning *et al.*, 2001; Guo *et al.*, 2002; Kondo *et al.*, 2002). This induction of MMP-9 could be due to the fact that NF-Kappa B can be induced by hypoxia (Muraoka *et al.*, 1997). The correlation between HIF-1α and MMP-9 raises the possibility that HIF-1α may have a regulatory role in the expression of this protease or it may reflect that both factors may be induced by similar stimuli such as hypoxia or EGFR (Cox *et al.*, 2000). More work is required to elucidate whether or not HIF-1α is involved in the regulation of MMP-9.

Immunopositivity of p53 is thought to represent mutated p53, as the half-life of mutated p53 is longer that that of wild type. Immunopositivity may also represent stabilised wildtype p53. The association between HIF-1α and p53 may reflect that there

is an interaction between these factors or that both are induced by hypoxia. HIF-1α has been reported to stablilise wildtype p53 and to induce apoptosis (Suzuki *et al.*, 2001). Conversely mutated p53 has been reported to increase HIF-1α (Blagosklonny *et al.*, 1998). The fact that there was no association between prognosis and co-expression of HIF-1α and p53 is consistent with activity of both these mechanisms (data not shown) (Cox *et al.*, 2000).

In vitro studies have shown that Bcl-2 and HIF-1α may regulate the activity of each other (Boyd *et al.*, 1994; Carmeliet *et al.*, 1998; Iervolino *et al.*, 2002). No association was found between Bcl-2 and HIF-1α making it unlikely that either consistently influences the expression of the other in NSCLC.

Previous studies have reported conflicting evidence as to whether HIF-1α expression is associated with MVC (Beasley *et al.*, 2002; Birner *et al.*, 2001; Birner *et al.*, 2001; Giatromanolaki *et al.*, 2001; Koukourakis *et al.*, 2002). Our study has shown no association between HIF-1α expression and MVC. The regulatory processes governing angiogenesis are complex and the predominant stimulus may vary in different parts of the tumour. For example hypoxia has been shown to be the dominant stimulus governing VEGF expression in perinecrotic areas of tumours but in the periphery of tumours, transforming growth factor (TGF)-β1 has been shown to be more important (Breier *et al.*, 2002). In our series the MVC and HIF-1α expression were exclusively evaluated in tumour edge sections (Cox *et al.*, 2000). Tumours with high TGF-β1 may have a stronger angiogenic stimuli resulting in higher MVC than those tumours that depend on hypoxia as an angiogenic stimulus.

In summary HIF-1α is widely expressed in NSCLC tissue. High levels of expression are associated with a poor prognosis independently of routinely used clinico-pathological variables. Although the numbers are relatively small in the high positive HIF-1α group the findings are in agreement with other studies of HIF-1α in solid tumours. The associations with EGFR, p53, MMP-9 and CA IX may be viewed as epiphenomena but support data from other studies that these factors are either involved in the regulation of, or are in part regulated by HIF-1α.

Tumour type	Stage	Treatment	Primary antibody	Pattern of expression	Cut point (%)	Number	P value survival	Ref
Cervical	Early	Surgery	Mab H1α67 NB 100-105	Nuclear	> 4 points	91	<0.0001†	(Birner *et al.*, 2000)
Cervical	Locally advanced	R/T	Mab H1α67 NB 100-105	Nuclear	2%	45	n/s	(Haugland *et al.*, 2002)
NSCLC	I-IIIA	Surgery	ESEE 122	Nuclear/ cytoplasmic	?	108	0.08†	(Giatromanolaki *et al.*, 2001)
Oropharyngeal	all	R/T +/- chemo	Mab H1α67 NB 100-105	Nuclear	Tertiles 10% +50%	98	0.001†	(Aebersold *et al.*, 2001)
Oesophageal	early	PDT+/- R/T	ESEE 122	Nuclear/ cytoplasmic	?	37	0.04†*	(Koukourakis *et al.*, 2001)
SCCHN	early	Surgery	ESEE 122	Nuclear	Positive or negative	79	0.027‡	(Beasley *et al.*, 2002)
SCCHN	Locally advanced	Chemo R/T	ESEE 122	Nuclear/ cytoplasmic	Mean value not quantified	75	0.05†	(Koukourakis *et al.*, 2002)
Oligodendroma		Surgery+/- chemo R/T	Mab H1α67 NB 100-105	Nuclear	>4 points	51	0.043†	(Birner *et al.*, 2001)
Breast	Lymph node positive	Surgery+/- chemo+/- tamoxifen	Mab H1α67 NB 100-105	Nuclear	quartiles	206	0.001†	(Schindl *et al.*, 2002)
Epithelial Ovarian	I-IV	Surgery adjuvant chemo	Mab H1α67 NB 100-105	Nuclear	quartiles	102	n/s	(Birner *et al.*, 2001)

Table 1. Summary of HIF-1α immunohistochemical studies.

*Response to photodynamic therapy not survival; ‡good prognosis; † poor prognosis. n/s: non-significant; ?: not stated in text.

Table 2. Previous immunohistochemistry performed on this series

Factor	Antibody	IHC kit
EGFR	Anti-EGFR clone EGFR.113 Novacastra, UK	ABC kit Dako, UK
MMP-9	Anti-MMP-9 clone 56-2A4, Chemicon, UK	ABC kit Dako, UK
Bcl-2	Anti-Bcl-2 clone 124, Dako, UK	ABC kit Dako, UK
P53	Anti-p53 NCL-p53-CM1, Novacastra, UK	ABC kit Dako, UK
CA IX	Anti-CA IX clone M75, gift from Professor Pastorek, Slovak Academy of Sciences	Envision kit, Dako, UK
MVC	Anti-CD 34 clone QB End/10, Novacastra, UK	ABC kit Dako, UK

Table 3. HIF-1α associations with clinico-pathological and biological variables

Prognostic Factor		Negative HIF-1α	Positive HIF-1α	χ^2 p value
EGFR	< Median	53	30	0.01
	≥ Median	39	49	
MMP-9	< 20%	51	26	0.003
	≥ 20%	36	48	
mCA IX	< Median	60	28	< 0.001
	≥ Median	30	50	
pCA IX	Negative	73	49	0.004
	Positive	16	30	
P53	< 20%	60	36	0.008
	≥ 20%	32	44	
Bcl-2	< 20%	64	49	0.25
	≥ 20%	28	31	
MVC	< Median	45	41	0.58
	≥ Median	47	36	
TN	Limited	69	32	< 0.001
	Extensive	23	48	
Stage	1	48	37	0.63
	2	24	26	
	3a	20	17	
T-Stage	1	18	13	0.042
	2	69	53	
	3/4	5	14	
N-Stage	0	51	41	0.48
	1	24	28	
	2	17	11	
Histology	Not Squamous	52	13	< 0.001
	Squamous	40	67	
Positive margins	No	85	72	0.58
	Yes	7	8	

Table 4. Univariate survival analysis

Variable		Hazard ratio	95% Confidence Intervals	P value
HIF-1α	< 60%	1		0.037
	≥ 60%	1.68	1.03-2.75	
pCA IX	Negative	1		0.031
	Positive	1.52	1.04-2.21	
mCA IX	< Median	1		0.11
	≥ Median	1.32	0.94-1.85	
MVC	<Median	1.0		
	≥Median	1.86	1.32-2.63	<0.001
TN	Limited	1		0.005
	Extensive	1.63	1.16-2.79	
MMP-9	Low	1		0.002
	High	1.76	1.23-2.5	
EGFR	< Median	1		0.72
	≥ Median	1.065	0.76-1.49	
P53	< 20%	1		0.57
	≥ 20%	1.10	0.79-1.55	
Stage	1	1		<0.001
	2	1.82	1.22-2.72	
	3A	2.63	1.74-3.99	
Bcl-2	< 20%	1		0.58
	≥ 20%	0.9	0.64-1.29	
Positive resections margins	Negative	1.0		0.1
	Positive	1.57	0.91-2.68	
Histology	Adenocarcinoma	1.0		0.33
	Large	0.84	0.39-1.82	
	Squamous	1.31	0.88-1.93	
	Other	1.8	0.64-5.06	
Grade	Well/mod diff	1.0		0.56
	Poor diff	1.1	0.79-1.54	

Table 5. Multivariate analysis for HIF-1α and routine clinico-pathological variables

Variable		Hazard ratio	95% confidence intervals	p value
Stage	I	1		<0.001
	II	1.74	1.15-2.64	
	IIIA	2.29	1.47-3.53	
HIF-1α	< 60%	1		0.006
	≥ 60%	2.05	1.23-2.44	
Gender	Female	1		0.027
	Male	1.60	1.06-2.44	

Table 6. Multivariate analysis for all clinico-pathological and biological variables

Variable		Hazard ratio	95% confidence intervals	p value
MVC	< Median	1		0.001
	≥ Median	1.92	1.3-2.83	
pCA IX	Negative	1		0.002
	Positive	1.96	1.28-3.01	
Platelets	Low	1		0.007
	High	1.72	1.16-2.56	
Gender	Female	1		0.012
	Male	1.77	1.13-2.78	
Stage	I	1		0.023
	II	1.56	1-2.44	
	IIIA	1.84	1.14-2.99	
MMP-9	Low	1		0.027
	High	1.56	1.05-2.3	

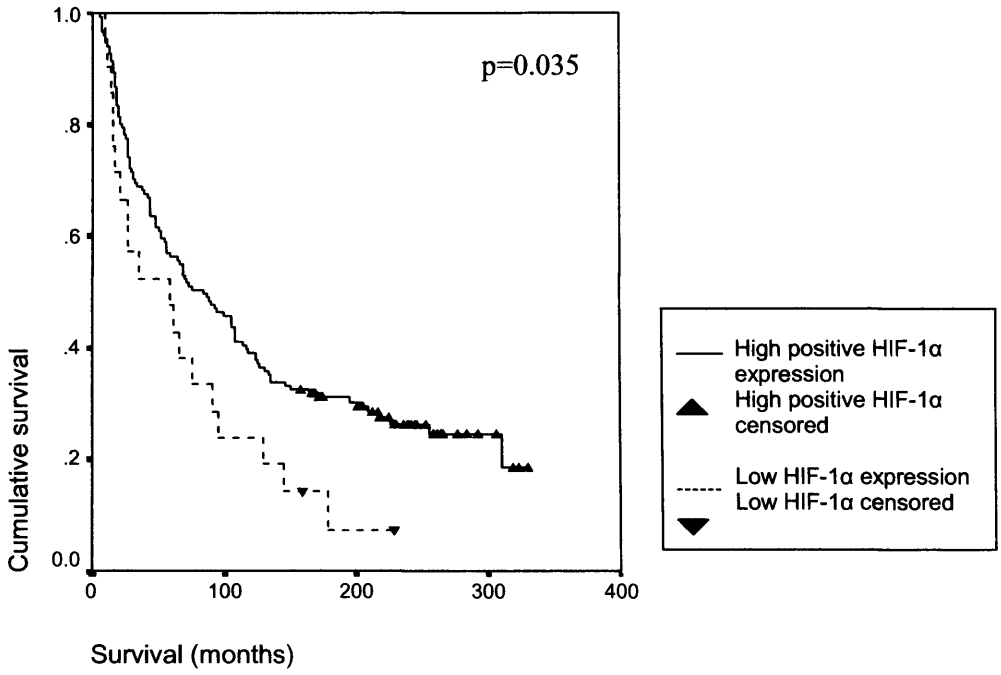

Figure 2. Kaplan Meier survival curve for HIF-1α expression in NSCLC

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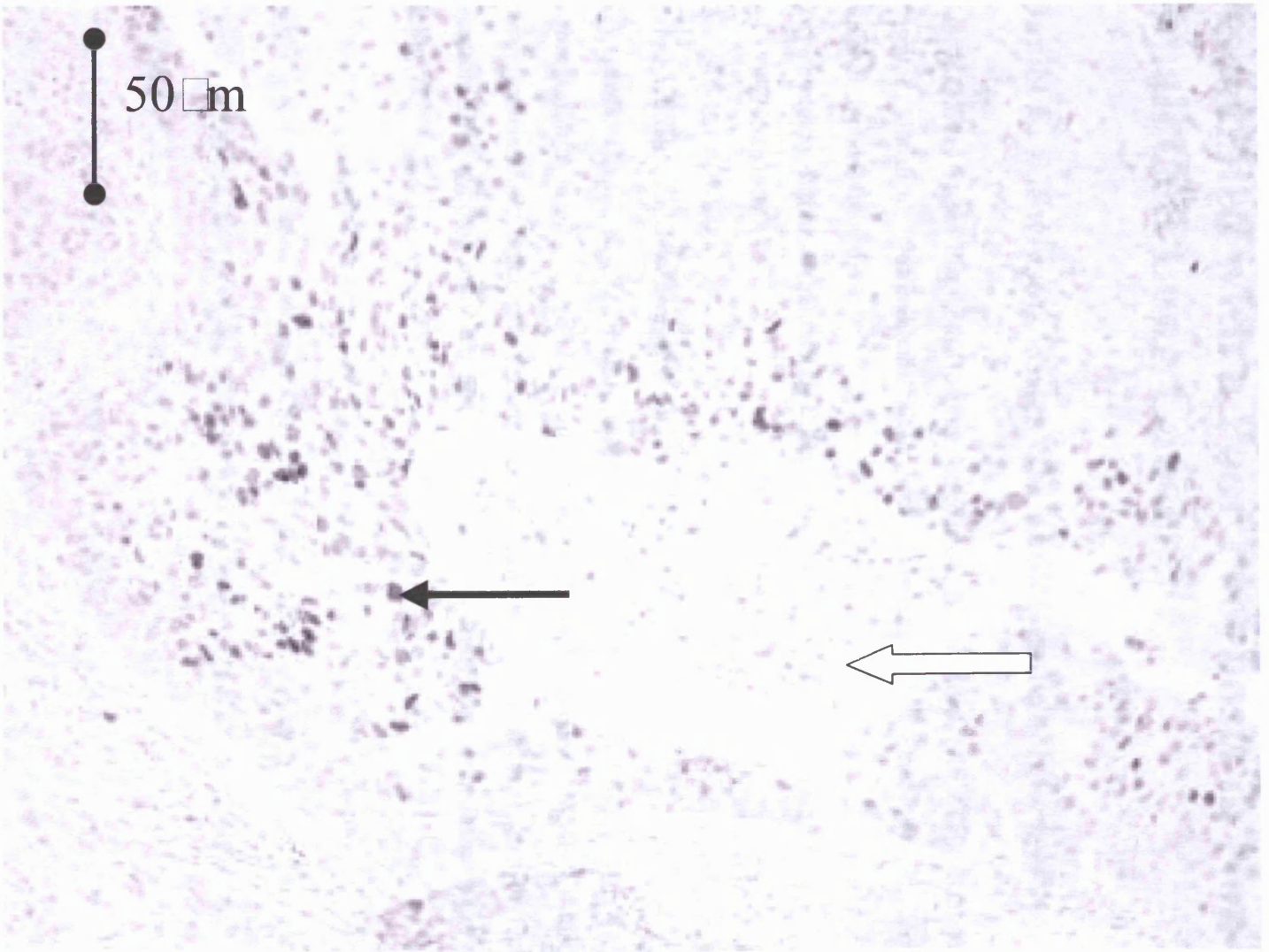
Figure 1



Co-expression of Epidermal Growth Factor Receptor with Related Factors is Associated with a Poor Prognosis in Non-Small Cell Lung Cancer

Daniel E. B. Swinson, #Giles Cox and *Kenneth J. O'Byrne

Cancer Centre, Queen Elizabeth Hospital, Edgbaston, Birmingham, B15 2TH. #Kingsmill Hospital, Mansfield, Nottinghham. NG1 1AA. *Department of Oncology, St. James Hospital, Dublin, Ireland.

Corresponding Author. Dr. Daniel E. B. Swinson

Tel.: +447958620077

Fax: +44-121-697-8428.

E-mail address: daniel.swinson@fsmail.net

Key words: CA IX, NSCLC, EGFR, MMP-9

Abbreviations used: NSCLC, non-small cell lung cancer; MMP-9, matrix metalloproteinase-9; Epidermal growth factor, EGF; EGFR, epidermal growth factor receptor; HIF, hypoxic inducible factor; CA, carbonic Anhydrase; transforming growth factor, TGF.

Summary In the region of 22-82% of Non-small cell lung cancer (NSCLC) tumours overexpress the epidermal growth factor receptor (EGFR). However EGFR expression does not reliably predict either prognosis or response to EGFR targeted therapy. This suggests that expression of EGFR alone is insufficient for tumour progression. Identification of patients with co-expression of EGFR and related factors may have greater clinical relevance. The data from 2 previous studies that explored the expression of EGFR and 2 possible downstream factors carbonic anhydrase (CA) IX and matrix metalloproteinase (MMP)-9 was analysed. EGFR expression was associated with both perinuclear (p) CA IX ($p<0.001$) and MMP-9 ($p<0.001$) expression. EGFR expression was not associated with prognosis. MMP-9 ($p=0.001$) and pCA IX ($p=0.03$) were associated with a poor prognosis. Multivariate analysis demonstrated that co-expression of EGFR with either MMP-9 or pCA IX conferred a significantly worse prognosis than either of these factors alone. The study population was divided into 3 groups: group 1 expression of EGFR without co-expression of MMP-9 or pCA IX, group 2 no expression of EGFR and group 3 co-expression of EGFR with pCA IX or MMP-9. Group 3 had a worse prognosis than either groups 1 or 2 ($p=0.0003$ and $p=0.027$, respectively) and group 1 had a better prognosis than group 2 ($p=0.036$). This data identifies 2 cohorts of EGFR positive patients with diametrically opposite prognoses. The group expressing EGFR and either or both MMP-9 and pCA IX may be more responsive to EGFR targeted therapies.

Epidermal growth factor receptor (EGFR) is a member of the c-erbB membrane receptor family and was first described in 1980 (Cohen et al., 1980). Since then there has been an increasing understanding of the importance of the role of EGFR in tumorigenesis. EGFR signaling promotes angiogenesis, cell proliferation, tumour invasion and inhibits tumor suppressor gene activity and apoptotic signaling (Abdollahi et al., 1999; Di Gennaro et al., 2003; Gildea et al., 2002; Hirata et al., 2002; Rosen et al., 2001; Westermark et al., 1982). In pre-clinical trials tumor cells with acquired resistance to hormonal or cytotoxic chemotherapy may be highly sensitive to EGFR targeted therapy (McClelland et al., 2001; Naruse et al., 2002; Parekh et al., 1997). Furthermore Gifitinib ('Iressa', ZD1839), a selective EGFR tyrosine kinase inhibitor, has been shown to have additive or even synergistic activities when co-administered with cytotoxic chemotherapy and radiotherapy (Aboud-Pirak et al., 1988; Baselga et al., 1993; Chinnaiyan et al., 2003; Fan et al., 1993; Sirotnak et al., 2000). Recently phase II clinical trials in heavily pre-treated non-small cell lung cancer (NSCLC) patients treated with Gifitinib have reported 10-18% radiological and ~ 40% symptomatic responses (Fukuoka et al., 2002; Kris et al., 2002). However recent large phase III studies demonstrated no clinical benefit when Gifitinib was added to standard cytotoxic chemotherapy in NSCLC (Giaccone et al., 2002; Johnson et al., 2002).

HER2/neu/c-erbB2 is a related member of the erbB family and has been identified as a target for treatment in breast cancer. Overall survival (OS) and response to HER 2/neu/c-erbB2 inhibition can be predicted by assessing the extent of HER2/neu/c-erbB2 expression (Slamon et al., 2001). Such a model has not been developed for EGFR targeted therapy in NSCLC. EGFR expression does not predict response to EGFR

targeted therapy (Bailey et al., 2003; Perez-Soler et al., 2001). Nor does it reliably predict OS, in that of the 17 immunohistochemical studies of EGFR expression in NSCLC only 2 have reported an association with prognosis (table 1) (Cornianu & Tudose, 1997; Cox et al., 2000; D'Amico et al., 1999; Fontanini et al., 1998; Fu et al., 1999; Greatens et al., 1998; Hirsch et al., 2003; Kanematsu et al., 2003; Mukohara et al., 2003; Ohsaki et al., 2000; Onn et al., 2003; Pastorino et al., 1997; Pfeiffer et al., 1996; Rusch et al., 1997; Selvaggi et al., 2002; Veale et al., 1987; Volm et al., 1993). There are important differences between HER2/neu/c-erbB 2 expression in breast cancer and EGFR expression in NSCLC that may contribute to this situation. EGFR, unlike HER2/neu/c-erbB2 activation requires the binding of specific ligands after which it dimerises either with another EGFR molecule, homodimerisation, or with another member of the c-erbB family, heterodimerisation. In this signaling pathway HER2/neu/c-erbB2 is the most frequent EGFR dimerisation partner (Cohen et al., 1981; Tzahar et al., 1996; Yarden & Ullrich, 1988). Hence expression of EGFR alone may not accurately represent EGFR activity. However co-expression of EGFR with its most common dimerisation partner also does not predict response to EGFR targeted therapy or OS and neither does co-expression with its most common ligand, transforming growth factor (TGF)α predict OS (27, (Cappuzzo et al., 2003; Kanematsu et al., 2003). Studies that are able to identify patients with activated EGFR may identify poor prognostic patients more likely to respond to EGFR targeted therapy. Indeed a small study of just 36 patients has reported that phosphorylated EGFR expression is associated with a poor prognosis (Kanematsu et al., 2003). In this review of data from 2 previous studies we have examined the prognostic impact of EGFR with related factors matrix metalloproteinase (MMP) 9 and carbonic

anhydrase (CA) IX (Cox et al., 2000; Swinson et al., 2003).

Matrix metalloproteinase (MMP)-9 is an enzyme that promotes angiogenesis and tumour invasion by breaking down the extracellular matrix. Increased MMP-9 expression is associated with EGFR expression in NSCLC and correlates with a poor prognosis (Cox et al., 2000). Epidermal growth factor (EGF) stimulation of EGFR positive cell lines has been demonstrated to induce MMP-9 expression and likewise inhibition of EGFR *in vivo* reduces tumour cell MMP-9 expression (O'Byrne et al., 2001; Perrotte et al., 1999). Carbonic anhydrase (CA) IX is a hypoxia inducible membrane bound enzyme involved in tumour cell pH balance. Membranous (m) CA IX expression has been postulated to be a novel marker of tumour hypoxia and peri-nuclear (p)CA expression correlates with a poor prognosis in NSCLC (Swinson et al., 2003). The transcription factor hypoxia inducible factor (HIF)-1 strictly regulates CA IX synthesis (Wykoff et al., 2000). EGF and hypoxia induce HIF-1α expression and transcriptional activity (Semenza, 2002). As such EGFR may enhance CA IX and MMP-9 induction.

MATERIALS AND METHODS

Ethics

The Leicester locoregional ethical committee granted ethical approval.

Literature search for studies investigating EGFR expression in NSCLC

Pubmed, Embase, Medline databases, the Cochrane library and ASCO annual meeting abstracts were searched using EGFR, Non-small cell lung cancer and immunohistochemistry as key words.

Immunohistochemistry

The specimens had previously been evaluated for the expression of EGFR, MMP-9 and CA IX. Standard immunohistochemical methods were employed using the anti-EGFR mouse Mab EGFR.113 (Novocastra Laboratories Ltd, Newcastle, UK.) (Cox et al., 2000), anti-MMP-9 mouse monoclonal antibody (Mab) 56-2A4 (Chemicon International Ltd.) (Cox et al., 2000), and anti CA IX antibody M75 (table 2) (Cox et al., 2000; Swinson et al., 2003).

Interpretation

The percentage of cells staining positively in each study was estimated using light microscopy. The cut points used to dichotomize the series in each study were predetermined. Twenty percent plus tumour cell staining was used as a cut point to define overexpression of EGFR (both cytoplasmic and membranous) and MMP-9 (cytoplasmic) (Cox et al., 2000). Two independent investigators, blinded from the other's results interpreted the slides and where a discrepancy was found a consensus was reached using a double-headed microscope. In the second study there were 3 distinct patterns of CA IX staining perinuclear (p), membranous (m) and cytoplasmic. The presence or absence of pCA IX staining was used as a cut point as this pattern of staining was an infrequent observation. Greater or equal to the median defined high membranous CA IX staining (Swinson et al., 2003). Two investigators blinded from each other's results again interpreted the staining. A third investigator adjudicated the result where discrepancies were found. The survival data from these studies was reviewed and up-dated (Cox et al.,

2000; Swinson et al., 2003).

Inclusion and exclusion criteria and patient follow up

A consecutive series of patients who had had NSCLC tumours resected with curative intent were considered for entry into the 2 studies. Patients were excluded if they had stage IV disease, survival of less than 61 days from time of operation to exclude the confounding factor of operative mortality and available tissue (Cox et al., 2000; Swinson et al., 2003). The final staging was based on the findings at surgery and the histopathology report. Hospital notes of the patients were reviewed and if necessary the local cancer registries or patient's General Practitioner were contacted to complete case follow up.

Statistical Analysis

The SPSS software system (SPSS for Windows Version 9.0) was used to perform the statistical analysis. The χ^2-test was used to analyze the associations between categorical variables. A p value of ≤ 0.05 was used as the level of significance. Overall survival as opposed to cancer specific mortality was used to avoid bias. Survival curves were plotted using the Kaplan-Meier method and log-rank test was used to assess the statistical significance of differences in survival. A Cox proportional hazards regression model was used to investigate whether co-expression of factors with EGFR significantly worsened outcome compared to expression of factors in isolation. A Cox proportional hazards regression model was also used to identify statistically significant differences in survival and estimate hazard ratios and 95% Confidence Intervals (CI). Co-variables were entered into the model if $p \leq 0.05$ and removed if $p \geq 0.1$.

RESULTS

Study Population

Two hundred and eighteen patients were considered for the 2 studies. Twenty four patients were excluded due to poor post operative survival and 13 patients were excluded as they were found to have pathological stage IV disease. Of the 181 cases initially available 179 were stained for EGFR expression, 177 were stained for CA IX expression and 169 were stained for MMP-9 expression. The reason for the reduced number of patients in the CA IX and MMP-9 series was tissue availability at the time of staining. In total there were 166 cases stained for all 3 markers. Of the 166 patients available for analysis, 115 (69.3%) were male and 51 (30.7%) were female. Eighty two (49.4%)

patients had stage I, 46 (27.7%) patients had stage II and 38 patients had stage IIIA (22.9%) disease. Forty seven (28.2%) patients had adenocarcinoma, 101 (60.8%%) patients had squamous carcinoma, 14 (8.4%%) patients had large cell carcinoma and 4 (2.4%) patients had tumours that were not characterized. The mean age at surgery was 65 years (S.D. 7.9 range 33.8-79.1). Positive resection margins were found in 15 patients. One patient had received adjuvant chemotherapy. Adjuvant radiotherapy was given to 17 patients, of whom 10 were stage IIIA, 6 were stage II and 1 was stage I.

One hundred and twenty six (75.9%) patients had died at the time of analysis and of these 18 (10.8%) were not cancer related. The duration of follow up from time of surgery was between 5 and 10 years.

Of the 15 patients that had been excluded due to lack of tissue there was no statistical difference in the stage ($p=0.72$), histology ($p=0.9$), sex ($p=0.56$) or adjuvant radiotherapy ($p=0.56$) distribution compared to the patients used in the survival analysis.

Associations between different patterns of CA IX staining and EGFR

Using the Chi square test mCA IX and pCA IX expression patterns were positively associated with EGFR expression. The association between pCA IX and EGFR expression was the strongest ($p<0.001$). All the pCA IX positive tumours expressed mCA IX. The association between the mCA IX group and EGFR was dependent on the pCA IX positive cases as it was lost when the pCA IX group was subtracted from the series ($p=0.93$) (table 3). PCA IX was therefore used in survival analyses for this review.

There was a positive trend for a positive association between pCA IX and MMP-9 expression ($p=0.09$) (table 3).

Univariate analysis using the log rank test for of EGFR related variables

Survival data for EGFR, pCA IX, and MMP-9 expression were updated for the study and there was no significant change in the previously reported outcomes *(data not shown)* (Cox et al., 2000; Swinson et al., 2003). MMP-9 (p=0.0015) and pCA IX (p=0.03) were associated with a poor prognosis and EGFR expression had no prognostic value (p=0.72) (figures 1-3).

Univariate analysis using Cox regression analysis for of EGFR related variables

Co-expression of EGFR with pCA IX, mCA IX or MMP-9 increased the hazard ratio value and strengthened the p value compared to these variables alone (table 4).

By entering these variables into a multivariate analysis model the increase in the hazard ratio for pCA IX and MMP-9 when co-expressed with EGFR was shown to be significant (tables 5 and 6). However this was not the case for co-expression of mCA IX.

Survival analysis for EGFR co-expression, no co-expression and no EGFR expression

In view of the associations between both pCA IX, MMP 9 and EGFR expression the study population was divided into 3 groups: Group 1 expression of EGFR in the absence of pCA IX and MMP-9; group 2 no expression of EGFR and group 3 co-expression of EGFR with either or both pCA IX or MMP-9. Group 3 had a worse prognosis than groups 1 or 2 and group 1 had a significantly better prognosis than group 2 (figure 4) (table 7). Using the Chi square test the pattern of EGFR expression (membranous, cytoplasmic or mixed)

did not differ between the groups.

Multivariate analysis

The clinicopathological factors stage, gender, positive margins and the use of adjuvant radiotherapy were entered into a multivariate model with the 3 groups. Stage, gender and EGFR groupings were independent prognostic variables (table 8).

DISCUSSION

A large number of studies have demonstrated *in vitro* and *in vivo* that EGFR is a positive tumour growth factor and agents targeted against EGFR in pre-clinical and clinical trials may induce tumour regression. However previous work on our cohort of NSCLC patients and the majority of other immunohistochemical studies in NSCLC have found that EGFR expression is not associated with outcome (Cornianu & Tudose, 1997; Cox et al., 2000; D'Amico et al., 1999; Fontanini et al., 1998; Fu et al., 1999; Greatens et al., 1998; Hirsch et al., 2003; Kanematsu et al., 2003; Mukohara et al., 2003; Ohsaki et al., 2000; Onn et al., 2003; Pastorino et al., 1997; Pfeiffer et al., 1996; Rusch et al., 1997; Selvaggi et al., 2002; Veale et al., 1987; Volm et al., 1993) A possible explanation could be that EGFR positivity as evaluated by IHC does not relate to EGFR signaling. In previous work we have found a strong association between EGFR and MMP-9 expression in patient tumour samples and *in vitro* EGFR activation induced MMP-9 mRNA expression in 4 of 5 NSCLC cell lines studied. Co-expression of EGFR and MMP-9 conferred a significantly worse prognosis than did MMP-9 on its own. This work suggested that expression of EGFR and downstream factors might reflect the EGFR related signaling

(Cox et al., 2000; O'Byrne et al., 2001).

We have previously reported two important patterns of CA IX staining in NSCLC, mCA IX, which has been proposed to be a marker of tumour cell hypoxia and pCA IX that is associated with a poor prognosis (Swinson et al., 2003). PCA IX was closely related to the mCA IX group as all pCA IX tumour cells expressed mCA IX and the majority of pCA IX positive cases (42/46) had high mCA IX expression. The association between EGFR and pCA IX was stronger than between mCA IX and EGFR. The latter relationship appeared to be dependent on the former as when the pCA IX subgroup was subtracted from the series the relationship between mCA IX and EGFR was lost. EGFR related signaling promotes expression of HIF-1α and its transcriptional targets such as VEGF via the phosphotidylinositol-3 kinase pathway (Clarke et al., 2001; Jiang et al., 2001; Tsuzuki et al., 2000; Zhong et al., 2000). These studies in conjunction with our observations suggest that EGFR may enhance hypoxic induction of CA IX by augmenting induction of HIF-1α.

The associations found between EGFR and either pCA IX and MMP-9 are grounds for the assumption that co-expression of EGFR with either or both of these factors represent cases with activated EGFR, whilst those that express only EGFR may represent cases with inactive EGFR. The polarization of prognosis depending on whether or not EGFR co-expresses with related factors suggests that the reason EGFR expression is not associated with prognosis is because a subgroup of cases have inactive EGFR. The difference in terms of prognosis between group 3 and the other 2 groups is easily explained by our assumption of EGFR activation. This assumption is also supported by a small study that has reported that phosphorylated EGFR is associated with a poor

prognosis in NSCLC (Kanematsu et al., 2003). The difference between groups 1 and 2 is less easily explained. One explanation could be that some cases in group 2 expressed pCA IX and MMP-9, both markers of a poor prognosis where as by definition no cases in group 1 expressed these factors. However subtraction of cases with either or both MMP-9 and pCA IX expression from group 2 did not alter the survival difference between the two groups *(data not shown)*. The inactivation of the EGFR in group 1 cases may represent cases where the receptor may be mutated and non-functional. Alternatively in these cases the receptor may be active but may stimulate pro-apoptotic pathways. Recent work has shown that cells expressing high levels of EGFR may undergo apoptosis particularly following exposure to EGF. Increasing the level of EGFR expression in a variety of cell types predictably leads to apoptosis, a process that requires an active tyrosine kinase but not EGFR autophosphorylation sites (Gulli et al., 1996; Hognason et al., 2001).

In summary we have demonstrated an association between EGFR and different patterns of CA IX expression and have previously demonstrated a similar relationship between EGFR and MMP-9. We have hypothesised that cases with co-expression of EGFR with either or both MMP-9 and pCA IX represent cases with activated EGFR. The activation of the EGFR promotes an aggressive NSCLC phenotype. By developing an assay to select cases with activated EGFR a cohort of patients may be identified that are highly responsive to anti-EGFR therapy. The implications of such a finding would have great clinical benefits.

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Figure Legend

Figure 1. Kaplan Meier Survival Curve for MMP-9 Expression in NSCLC

Figure 2. Kaplan Meier Survival Curve for EGFR Expression in NSCLC

Figure 3. Kaplan Meier Survival Curve for Perinuclear CA IX Expression in NSCLC

Figure 4. Survival curves for EGFR expression alone (group 1), EGFR negative (group 2) and Co-expression of EGFR with either or both MMP-9 and CA IX (group 3).

Figure 1. Kaplan Meier Survival Curve and Log Rank P value for MMP-9 Expression in NSCLC

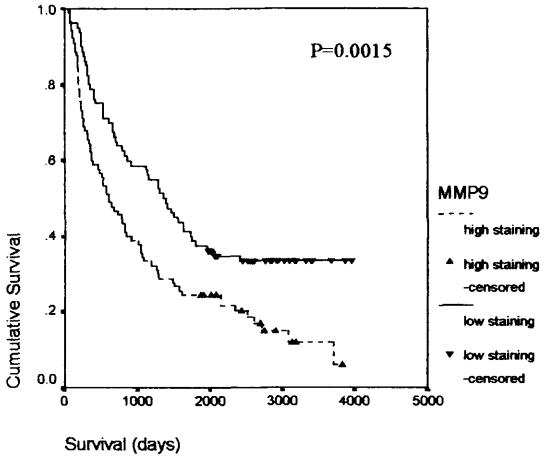

Figure 2. Kaplan Meier Survival Curve and Log Rank P value for Perinuclear CA IX Expression in NSCLC

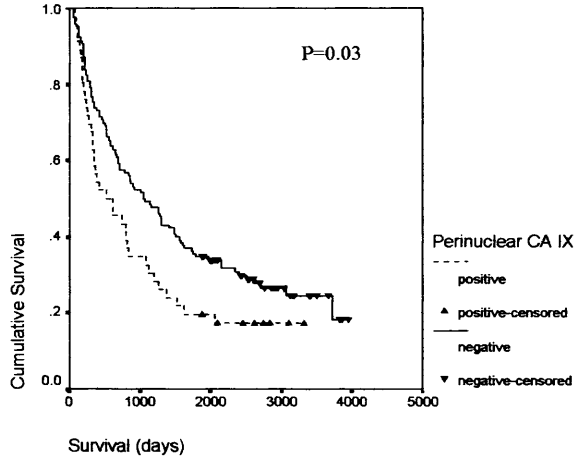

Figure 3. Kaplan Meier Survival Curve and Log Rank P value for EGFR Expression in NSCLC

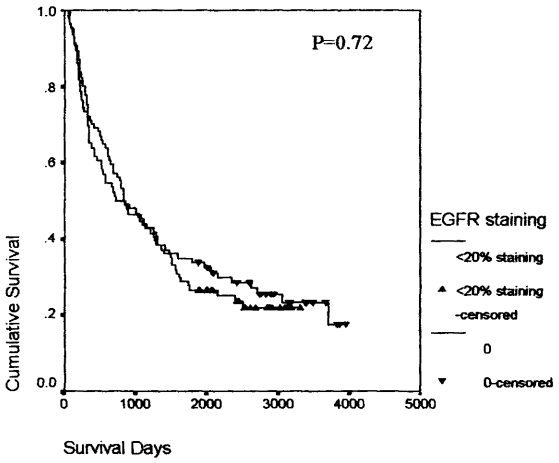

Figure 4.

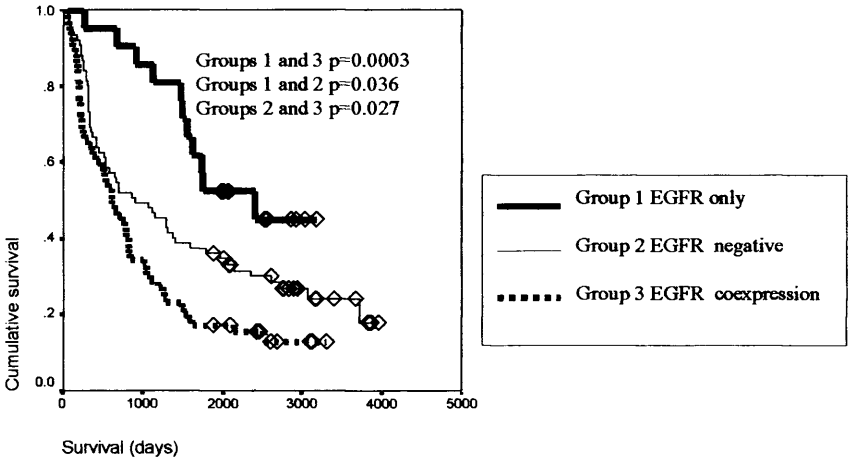
Groups 1 and 3 p=0.0003
Groups 1 and 2 p=0.036
Groups 2 and 3 p=0.027
Cumulative survival
1.0
.8
.6
.4
.2
0.0
0
1000
2000
3000
4000
5000
Survival (days)
Group 1 EGFR only
Group 2 EGFR negative
Group 3 EGFR coexpression

Table 1. Immunohistochemical studies of EGFR expression in NSCLC

No.	Staining pattern	Stage	Cut point	Percentage overexpressed	P value	EGFR Antibody	Ref
169	m and c	I-IIIA	≥ 20%	56%	0.17	Novacastra Labs EGFR.113	(Cox et al., 2000)
96		I-IIIA	≥ 20%	32%	n/s		(Rusch et al., 1997)
186	M	I-IV	0%, 0<80%, ≥80%	14%, 31%, 55%	0.9	R1, Amersham	(Pfeiffer et al., 1996)
515		I	<10%	50%, 47%	0.25	31G7, Triton	(Pastorino et al., 1997)
290		I-IV		43%	0.02υ		(Ohsaki et al., 2000)
158		I-IIIA	>0%	66%	0.36	Sigma 014H4819	(Fu et al., 1999)
77	m and c	I-IV	0-+++		p<0.05 κ	EGFR1	(Veale et al., 1987)
121					n/s		(Volm et al., 1993)
195		I-IIIA	>45%	53%/ 47%	0.80	Anti-EGFR Triton	(Fontanini et al., 1998)
408		I			n/s		(D'Amico et al., 1999)
183	M	I-III	Tertiles	38%/ 25%/ 37%	0.22	Zymed Labs No. 28-0005	(Hirsch et al., 2003)
					υ		(Cornianu & Tudose, 1997)
101		I-III	++/ +++ intensity ≥5%	35%/ 66%	n/s	Zymed Labs clone 31G7	(Greatens et al., 1998)
60		I-III	≥30%	22%/78%	0.6	Zymed Labs clone 31G7	(Mukohara et al., 2003)
36		I-III		81%/19%	n/s		(Kanematsu et al., 2003)
98	M	I	≥10%	61%/39%	n/s		(Onn et al., 2003)
130		I-III	≥10%	37%/63%	P<0.01υ	Oncogene Ab-1	(Selvaggi et al., 2002)

S=significant, n/s=not significant, υ associated with a poor prognosis, κ associated with stage survival analysis not performed

Table 2. Immunohistochemistry techniques and antibodies

	EGFR	MMP-9	CA IX
Antigen retrieval	Pressure cooking 2 mins	Pressure cooking 2 mins	No antigen retrieval
Blocking serum	Rabbit	Rabbit	Human
Primary antibody	Novacastra EGFR.113	Chemicon 56-2A6	M75
Dilution	1:20	1:100	1:50
Incubation	Overnight 4°C	Overnight 4°C	30 minutes at 20°C
Secondary antibody	Rabbit anti-mouse Ig (Dako)	Rabbit anti-mouse Ig (Dako)	Goat anti-mouse Ig (Envision Kit, Dako)
Dilution	1:400	1:400	Neat
Buffer	100mmol Tris, 300mmol NaCl TBS, pH 7.65	100mmol Tris, 300mmol NaCl TBS, pH 7.65	100mmol Tris, 300mmol NaCl TBS, pH 7.65
IHC kit	ABC (Dako)	ABC (Dako)	Envision (Dako)
Cut point for categorical analysis	≥20%	≥20%	≥5% (mCA IX) >0% (pCA IX)
Reference	(Cox et al., 2000)	(Cox et al., 2000)	(Swinson et al., 2003)

Table 3. Frequency table for pCA IX, EGFR and MMP-9 expression

Factor		Negative pCA IX	Positive pCA IX	χ^2 p value
EGFR	< 20%	73	11	<0.001
N=176	≥ 20%	58	34	
MMP-9	Low	61	18	0.09
N=166	High	59	28	
		Low mCA IX	High mCA IX	
EGFR	< 20%	53	31	0.009
N=176	≥ 20%	40	52	
pCA IX positive cases subtracted		Low mCA IX	High mCA IX	
EGFR	< 20%	50	22	0.79
N=130	≥ 20%	39	19	

Table 4. Univariate survival of EGFR related biological variables using Cox regression analysis

Prognostic factor		N	Hazard ratio	95% CI	p-value
EGFR	<20%	86	1		0.64
	≥20%	93	1.08	0.77–1.51	
		179			
MMP-9	<20%	80	1		0.001
	≥20%	89	1.79	1.26-2.55	
		169			
EGFR/ MMP-9	-ve	61	1.00		<0.0001
Co-expression	+ve	107	2.19	1.54-3.1408	
		168			
PCA IX	Positive	46	1.0		0.044
	Negative	131	1.50	1.03–2.19	
		177			
EGFR/ pCA IX	-ve	142	1		0.003
Co-expression	+ve	34	1.86	1.24-2.80	
		176			

Table 5. Cox regression model for EGFR/ MMP-9 co-expression

Variable		Harzard Ratio	95% Confidence Intervals	p value
MMP-9	< 20%	1.0		0.792
	≥ 20%	0.93	0.54-1.59	
EGFR	< 20%	1.0		0.015
	≥ 20%	0.52	0.31-0.88	
MMP-9/ EGFR	Negative	1.0		<0.001
Co-expression	Positive	3.55	1.73-7.26	

Table 6. Cox regression model for EGFR/ pCA IX co-expression

Variable		Hazard Ratio	95% Confidence Intervals	p value
PCA IX	Negative	1.0		0.781
	Positive	0.90	0.42-1.89	
EGFR	<20%	1.0		0.172
	≥20%	0.75	0.50-1.13	
PCA IX/ EGFR	Negative	1.0		0.05
Co-expression	Positive	3.55	1.0-5.85	

Table 7. Cox regression analysis for pCA IX, MMP-9 and EGFR Co-expression

		Hazard ratio	95% CI	p value
N=21	No EGFR expression	1.0		0.0002
N=75	EGFR only	0.49	0.93-0.26	0.031
N=70	EGFR/MMP-9 or pCA IX co expression	3.25	1.70-6.20	0.0004

Table 8. Multivariate analysis of clinicopathological variables and EGFR groupings

Number	Variable	Hazard ration	95% CI	P value
82	Stage 1	1.0		0.001
46	Stage 2	1.55	1.02-2.37	0.04
38	Stage 3A	2.26	1.47-3.49	0.0002
51	Female	1.0		
115	Male	1.76	1.17-2.66	0.007
21	EGFR only	1.0		0.0003
75	No EGFR	2.11	1.1-4.08	0.025
70	EGFR co-expression	3.26	1.69-6.27	0.0004

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